Engineering an Improved Adenovirus Packaging Cell Line

by

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ABSTRACT

Recombinant human adenovirus (Ad) vectors are being extensively explored for their use in gene therapy and recombinant vaccines. Ad vectors are attractive for many reasons, including the fact that (1) they are relatively safe, based on their use as live oral vaccines, (2) they can accept large transgene inserts, (3) they can infect dividing and postmitotic cells, and (4) they can be produced to high titers. However, there are also a number of major problems associated with Ad vectors, including transient foreign gene expression due to host cellular immune responses, problems with humoral immunity, and the creation of replication competent adenoviruses (RCA).

Most Ad vectors contain deletions in the E1 region that allow for insertion of a transgene. However, the E1 gene products are required for replication and thus must be supplied in *trans* by a helper cell line that will allow for the growth and packaging of the defective virus. For this purpose the 293 cell line (Graham *et al.*, 1977) is used most often; however, homologous recombination between the vector and the cell line often results in the generation of RCA. The presence of RCA in batches of adenoviral vectors for clinical use is a safety risk because they may result in the mobilization and spread of the replication-defective vector viruses, and in significant tissue damage and pathogenicity. The present research focused on the alteration of the 293 cell line such that RCA formation can be eliminated.

The strategy to modify the 293 cells involved the removal of the first 380 bp of the adenovirus genome through the process of homologous recombination. The first step towards this goal involved identifying and cloning the left-end cellular-viral junction from 293 cells to assemble sequences required for homologous recombination. Polymerase chain reaction (PCR) was performed to clone the junction, and the clone was verified through sequencing. The plasmid PAM2 was then constructed, which served as the targeting cassette used to modify the

293 cells. The cassette consisted of (1) the cellular-viral junction as the left-end region of homology, (2) the *neo* gene to use for positive selection upon tranfection into 293 cells, (3) the adenoviral genome from bp 380 to bp 3438 as the right-end region of homology, and (4) the HSV-*tk* gene to use for negative selection.

The plasmid PAM2 was linearized to produce a double strand break outside the region of homology, and transfected into 293 cells using the calcium-phosphate technique. Cells were first selected for their resistance to the drug G418, and subsequently for their resistance to the drug Gancyclovir (GANC). From 17 transfections, 100 pools of $G418^r$ and $GANC^r$ cells were picked using cloning rings and expanded for screening. Genomic DNA was isolated from the pools and screened for the presence of the 380 bps using PCR. Ten of the most promising pools were diluted to single cells and expanded in order to isolate homogeneous cell lines. From these, an additional 100 $G418^r$ and $GANC^r$ foci were screened. These preliminary screening results appear promising for the detection of the desired cell line. Future work would include further cloning and purification of the promising cell lines that have potentially undergone homologous recombination, in order to isolate a homogeneous cell line of interest.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

µg	microgram
µl	microliter
µm	micrometer
Ad	adenovirus
ARD	Acute Respiratory Disease
BAV	bovine adenovirus
bp	base-pair
cAMP	cyclic adenosine monophosphate
cDNA	copy DNA
CAR	coxsackie and adenovirus receptor
CAV	canine adenovirus
CPE	cytopathic effect
CF	cystic fibrosis
CFTR	cystic fibrosis transmembrane conductance regulator
CMV	cytomegalovirus
CR1	conserved region 1
CR2	conserved region 2
CTL	cytotoxic T lympocytes
Da	dalton
DBP	DNA binding protein
dCMP	deoxycytidyl monophosphate
dCTP	deoxycytidyl triphosphate
DNA	deoxyribonucleic acid
EBV	epstein barr virus
EDS	egg drop syndrome virus
FDA	food and drug administration
FMDV	foot and mouth disease virus
GANC	gancyclovir
GDNF	glial cell-line derived neurotropic factor
HEK	human embryonic kidney

HIV	human immunodeficiency virus
HPV	human papilloma virus
HSV	herpes simplex virus
ITR	inverted terminal repeats
kbp	kilo base-pair
kDa	kilodalton
MHC	major histocompatibility complex
min	minutes
MOI	multiplicity of infection
mRNA	messenger RNA
neo	neomycin
NFI	nuclear factor one
NFII	nuclear factor two
NFIII	nuclear factor three
nm	nanometer
nt	nucleotide
OAV	ovine adenovirus
OTC	ornithine-transcarbamylase
PFU	plaque-forming unit
PGK	phosphoglycerate kinase
PP	protein phosphatase
PSG4	pregnancy-specific β-1-glycoprotein
pTP	precursor to the terminal protein
RB	retinoblastoma
RCA	replication competent adenoviruses
RNA	ribonucleic acid
RSV	respiratory syncytial virus
tk	thymidine kinase
TNF	tumor necrosis factor
TNFβ	tumor necrosis factor beta
TP	terminal protein
VSV	vesicular stomatitis virus

1. INTRODUCTION AND LITERATURE REVIEW

1.1 HISTORY AND EPIDEMIOLOGY

Adenoviruses were independently discovered by two groups of researchers (Rowe *et al.*, 1953, Hilleman and Werner, 1954). While attempting to establish cell lines from the adenoids removed from a number of children, Rowe and his colleagues discovered a cytopathic agent that was causing the human adenoids to undergo spontaneous degeneration. These agents were originally proposed to be designated as the "adenoid degeneration agent" or "A.D. agent" (Rowe *et al.*, 1953). Almost simultaneously, Hilleman and Werner were investigating an epidemic of acute respiratory illness among military recruits. They isolated a previously unidentified microbial agent from the throat washings of a patient with primary atypical pneumonia, and found that the agent multiplied in human cell tissue cultures. The viruses isolated by the two groups were soon shown to be the same virus.

Adenovirus infections can cause a number of clinical manifestations including respiratory infections, conjunctivitis, hemorrhagic cystitis, and gastroenteritis (Table 1). Infants, children, military recruits and immunocompromised individuals are the most susceptible to adenoviral infections (Rubin and Rorke, 1988). Of all the adenovirus serotypes, Ad7 is the most severe cause of respiratory infections, and Ad8 is responsible for the most severe outbreaks of keratoconjunctivitis (reviewed in Wadell, 1994). Adenoviral respiratory infections, which are extremely common in military recruits, lead to a condition referred to as ARD (Acute Respiratory Disease). ARD is most commonly caused by Ad4, Ad7 and occasionally Ad3, and often occurs under the conditions of fatigue and crowding experienced by military recruits (reviewed in Horwitz, 1990a). After 25 years of successful control through immunization, respiratory infections due to Ad7 and Ad3 have reemerged to threaten the health of young adults

in the military (Ryan *et al.*, 2002). Less commonly, adenoviral infections can be associated with central nervous system infections including aseptic meningitis, meningoencephalitis and encephalitis (Rubin and Rorke, 1988).

Table 1. Diseases caused by adenoviral infections (reproduced from Horwitz, 1990a)

Disease	Individuals Most at Risk	Principal Serotypes
Acute febrile pharyngitis	Infants, young children	1, 2, 3, 5, 6, 7
Pharyngoconjunctival fever	School-age children	3, 7, 14
Acute respiratory disease	Military recruits	3, 4, 7, 14, 21
Pneumonia	Infants, young children	1, 2, 3, 7
Pneumonia	Military recruits	4, 7
Epidemic keraconjunctivitis	Any age group	8, 11, 19, 37
Pertussis-like syndrome	Infants, young children	5
Acute hemorrhagic cystitis	Infants, young children	11, 21
Gastroenteritis	Infants, young children	40, 41
Hepatitis	Infants and children with liver transplants	1,2,5
Persistence of virus in urinary tract	AIDS, other immunosupression, and bone marrow transplant recipients	34, 35

1.2 CLASSIFICATION

Adenoviruses belong to the family Adenoviridae, which is divided into two genera, *Mastadenovirus* and *Aviadenovirus*. The *Mastadenovirus* genus includes more than 80 serologically distinct types of mammalian viruses including simian, bovine, equine, porcine, canine and 47 types of human adenoviruses (reviewed in Doerfler, 1994). In 1993, two additional human serotypes were recognized (Schnurr and Dondero, 1993), and recently candidate Ad serotypes 50 and 51 were isolated from AIDS patients (De Jong *et al.*, 1999). The genus *Aviadenovirus* contains viruses isolated from avian species including fowl, turkey, goose, pheasant and duck. There is no shared family-specific antigen among the Adenoviridae; however, the genus *Mastadenovirus* does share a genus-specific antigen (reviewed in Wadell, 1994). Recently, a third genus has been proposed which would contain bovine adenoviruses

(BAVs) 4-8, Ovine adenovirus (OAV) isolate 287 and egg drop syndrome (EDS) virus. The proposed name of the genus is *Atadenovirus*, referring to the high genomic AT content of the proposed members (Benko and Harrach, 1998; Dan *et al.*, 1998).

The 47 human adenoviruses were originally classified into four groups on the basis of hemagglutination patterns with rat and rhesus monkey red blood cells (reviewed in Horwitz, 1990b). Once it was discovered that human Ad12 could induce tumors in hamsters (Trentin *et al.*, 1962), it was then proposed that a new classification system should be used to divide the human adenoviruses into genera based on their oncogenesis in newborn hamsters (reviewed in Wadell, 1994). Currently the human adenoviruses are divided into 6 genera, A-F, based on their hemagglutination patterns, oncogenicity in rodents, DNA base composition and homology, restriction endonuclease patterns of the genome, viral polypeptide patterns and length of fibers (Table 2).

Table 2. Properties of human adenovirus serotypes of subgenera A-F (reproduced from Wadell, 1994).

Subgenus	Serotype	G+C (%)	Hemagglutination Pattern	Length of Fibers (nm)	Oncogenicity in Newborn Hamsters	Tropism Symptoms
A	12,18,31	48	IV	28-31	High	Cryptic enteric infection
B	3,7,11,14, 16,21,34, 35	51	I	9-11	Weak	Respiratory Disease, Persistent infections of the kidneys
C	1,2,5,6	58	III	23-31	Nil	Respiratory disease persists in lymphoid tissues
D	8,9,10,13, 15,17,19, 20,22 to 30,32,33, 36,37,38, 39,42 to 47	58	II	12-13	Nil	Keratoconjunctivitis
E	4	58	III	17	Nil	Conjunctivitis, Respiratory disease
F	40,41	52	IV	28-33	Nil	Infantile diarrhea

1.3 VIRAL PARTICLE AND GENOME

The adenovirus particle is a non-enveloped icosahedron that is 65-80 nm in diameter depending on the serotype (Lee *et al.*, 1995). The icosahedral capsid is composed of 252 capsomeres, including 240 hexons and 12 pentons. The 12 pentons occupy the vertices of the virion, and a knobbed fiber projects from each of these vertices (reviewed in Horwitz, 1990b). The fiber is a trimeric protein composed of; (1) an amino-terminal tail, which tethers the fiber to the virus via the penton base; (2) a shaft, which gives the fiber its long length; and (3) a carboxy-terminal knob, which contains the domains for receptor binding as well as for trimerization (reviewed in Kovesdi *et al.*, 1997). The length of the fiber varies with the adenovirus serotype (Russell, 1994). Each penton capsomere is surrounded by five hexons, and thus the name penton is derived from this arrangement. Each hexon is similarly surrounded by six capsomeres (reviewed in Horwitz, 1990b).

The virion is composed of a number of polypeptides (Figure 1). The hexon subunit is composed of three molecules of polypeptide II (120 kDa) which are held together by noncovalent interactions (reviewed in Horwitz, 1990b). Polypeptides VI (24 kDa), VIII (13 kDa), and IX (12 kDa) are associated with the hexon, and likely play a role in formation of the virion's outer shell (reviewed in Doerfler, 1994). Polypeptide IIIa (66 kDa) is associated with the hexons that are in direct contact with pentons. Each penton subunit is formed by five molecules of polypeptide III (85 kDa), and the associated fiber is known to be composed of three molecules of polypeptide IV (62 kDa) (reviewed in Horwitz, 1990b). Polypeptides V (48.5 kDa) and VII (18.5 kDa) are associated with the viral DNA in the core structure and are very rich in arginine, which neutralizes many of the negative charges on the DNA (Levy *et al.*, 1994). Also residing within the core is a 55 kDa terminal protein (TP), which is covalently linked to the viral DNA and plays a role in the initiation of DNA replication. Another core protein is the μ protein

(4 kDa), which is rich in arginine and histidine, but currently its function is unknown (reviewed in Horwitz, 1990b).

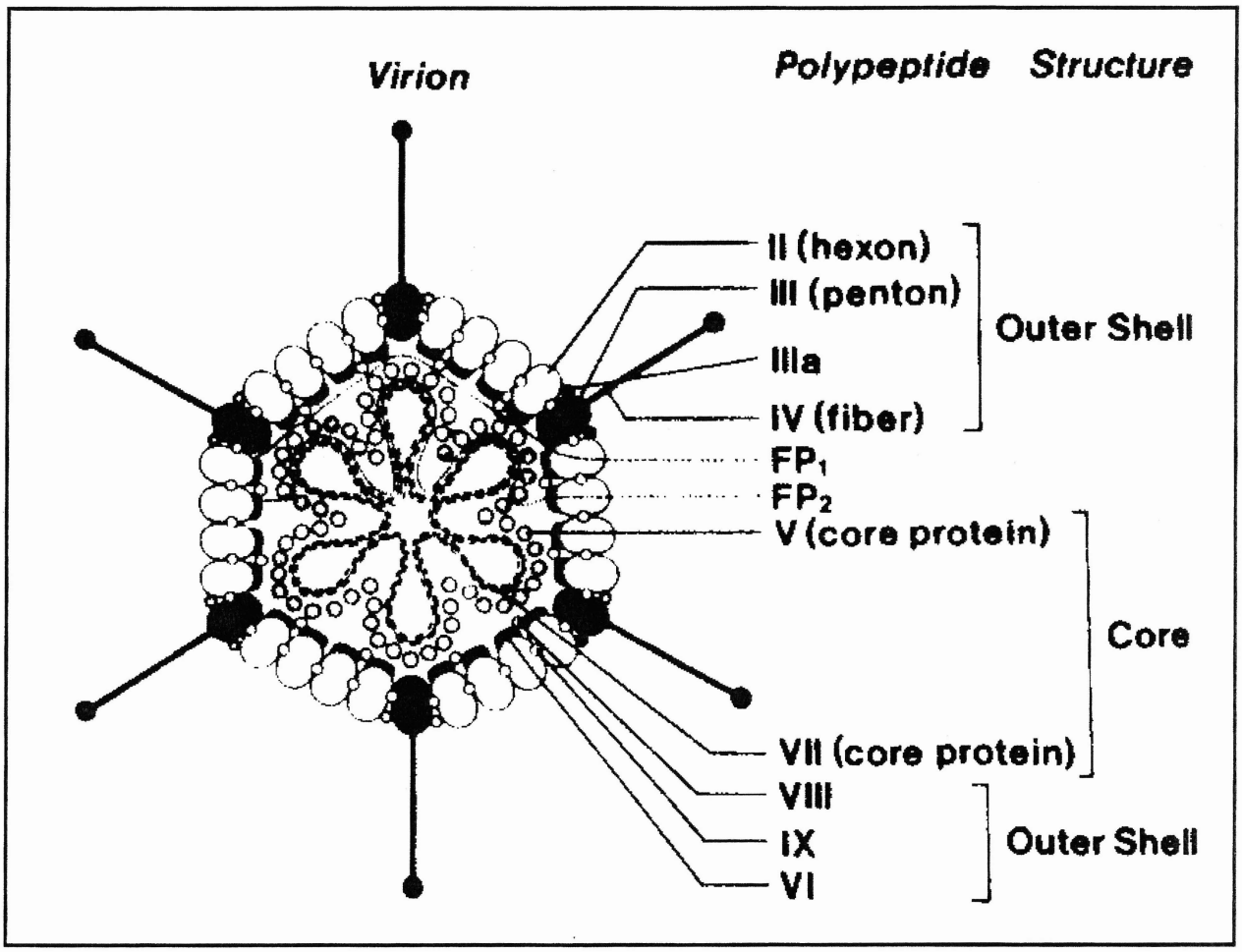


Figure 1. A schematic diagram of an adenovirus particle (reproduced from Doerfler, 1994).

Adenoviral DNA is linear and double-stranded, and has a size of 20 to 24 x 10^6 Da depending on the serotype (Levy *et al.*, 1994). The Ad DNA is approximately 11 μm in length or 36 kbp, and has inverted terminal repeats of 100- to 140- base pairs in length, again depending on the serotype (reviewed in Horwitz, 1990b). These terminal repeats allow the DNA, when

single-stranded, to form panhandle-shaped molecules (Levy *et al.*, 1994). The GC content of the DNA also varies with serotype, and is usually the highest for the most oncogenic serotypes. The DNA also has the unique feature of a 55-kDa terminal protein covalently linked to dCMP at each 5′ end of the linear genome, which is involved in DNA replication (reviewed in Horwitz, 1990b).

1.4 VIRAL LIFE CYCLE

1.4.1 Virus Entry

Adenoviral entry into host cells is both efficient and rapid, with about 40% of bound virus particles able to release their DNA inside the cell (Greber *et al.*, 1993). Adenoviruses are known to enter their host cell by receptor mediated endocytosis, which ultimately results in the delivery of the viral DNA to the host nucleus. Initially the fiber attaches to a receptor on the surface of the host cell, and two cellular receptors for the fiber have been reported. One receptor is termed the coxsackie and adenovirus receptor (CAR) because it functions as a receptor for both adenovirus and coxsackie virus, and it contains two immunoglobulin domains (Bergelson *et al.*, 1997; Tomko *et al.*, 1997). It has recently been shown that the IgV domain is necessary and sufficient for binding, and no additional membrane components are required to confer high-affinity binding to the Ad5 fiber knob (Kirby *et al.*, 2000). The α2 domain of major histocompatibility complex (MHC) class I has also been reported to mediate adenovirus attachment via the fiber (Hong *et al.*, 1997), however its role remains uncertain. The secondary cell surface receptor has been identified as the vitronectin binding integrins, which bind to the penton base of the virus and help to mediate the endocytosis (Wickham *et al.*, 1993). After binding to the receptors, the viruses are internalized via receptor-mediated endocytosis in clathrin-coated pits. In these endosomes, the pH falls to 5.5, leading to the rupture of the

endosome and the release of the virion into the cytoplasm (reviewed in Lee *et al.*, 1995). Adenoviruses undergo multiple sequential uncoating steps as they move from the cell surface to the nuclear membrane (Figure 2). First, the fiber and polypeptide IIIa dissociate 10-12 min after penetration, and the penton base is lost 3-5 min later. Next, the proteins linking the DNA to the inside surface of the capsid are degraded or shed, including proteins VI and VIII. Lastly, the capsid stabilizing minor proteins (IX, IIIa) are lost in endosomes. The uncoating process starts immediately upon endocytic uptake, and results with the entrance of dissociated hexon proteins and DNA into the nucleus (Greber *et al.*, 1993).

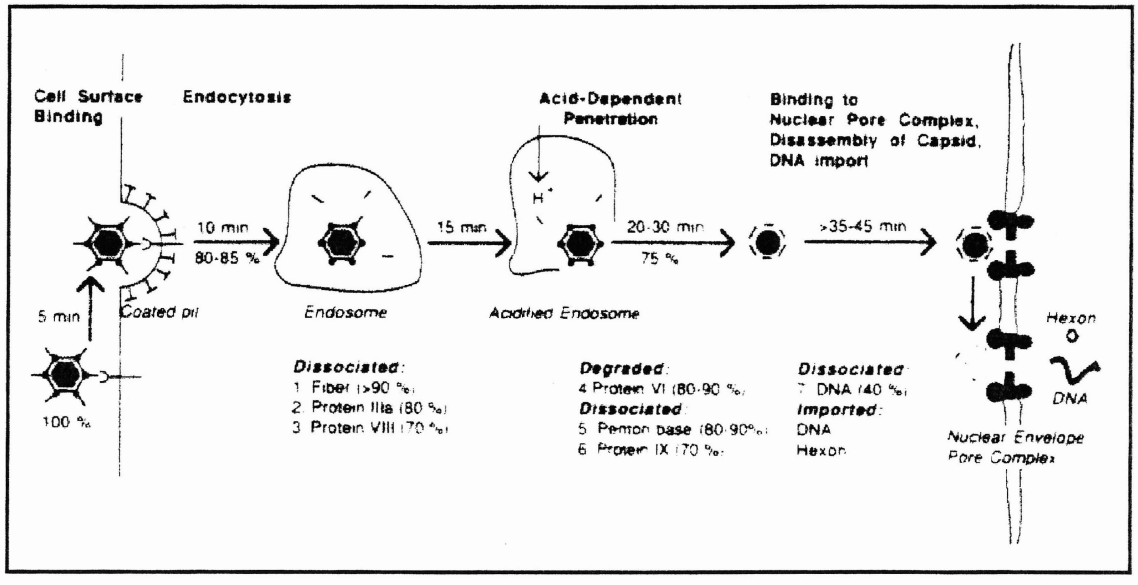


Figure 2. Sequential steps involved in the uncoating of adenoviruses during cell entry (reproduced from Greber *et al.*, 1993).

1.4.2 Early Gene Expression

The adenovirus replicative cycle is divided into early and late phases, with the early phase occurring before the onset of viral DNA replication, and the late phase occurring after viral DNA replication begins. Early cytoplasmic mRNAs are complementary to seven noncontiguous regions on the viral DNA, starting from six different promoters. The early transcripts are divided into "immediate early" (E1A), "delayed early" (E1B, E2A, E2B, E3 and E4), and "intermediate" (IVa2, IX) (reviewed in Horwitz, 1990b). Each region appears to contain a group of genes coding for proteins having related functions (reviewed in Lee *et al.*, 1995). The genes are transcribed by the host's DNA-dependent RNA polymerase II (reviewed in Doerfler, 1994). When considering the double-stranded linear genome of adenoviruses, the upper strand is termed the right or right-transcribed strand, while the lower is the left or left-transcribed strand. The right strand contains the E1 region at 1.3-11.2 map units and the E3 region from 76.6-86.2 map units. The left strand contains the E4 region at 96.8-91.3 map units, the E2A region from 67.9-61.5 map units and the E2B region from 29-14.2 map units (Figure 3) (reviewed in Horwitz, 1990b).

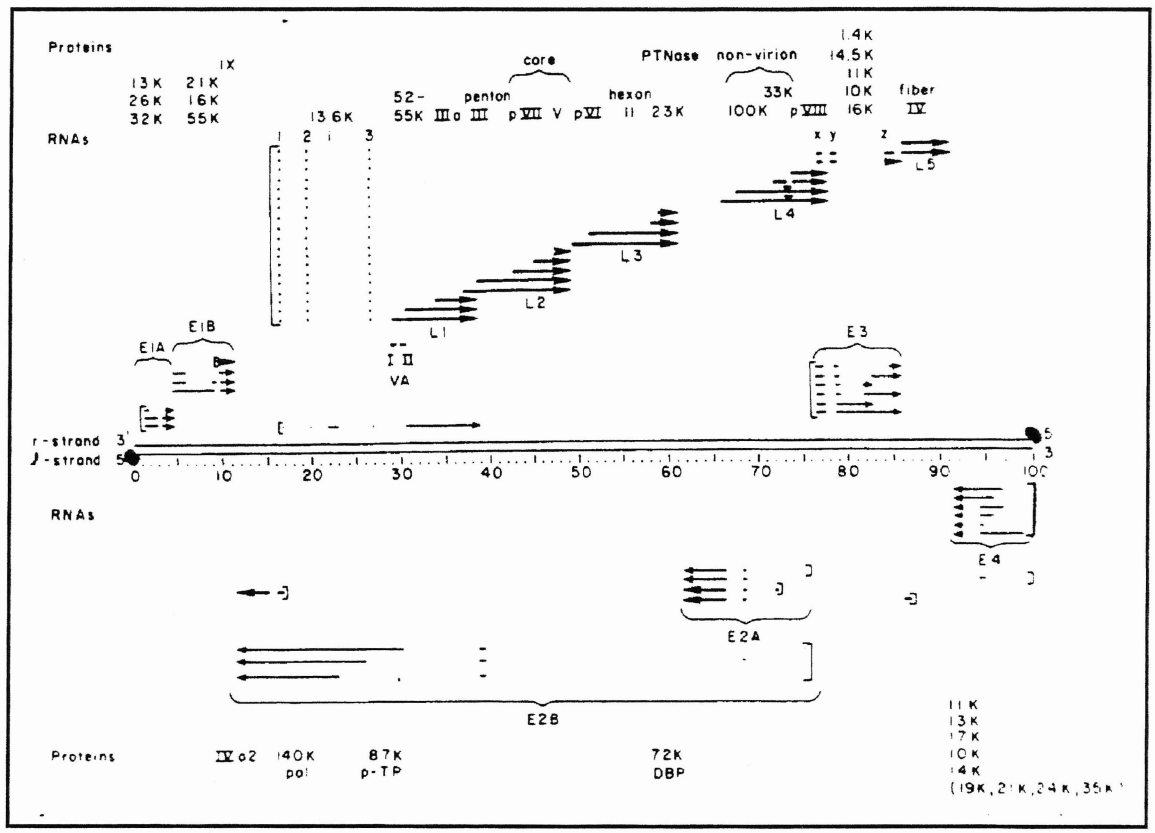


Figure 3. Transcription and translation map of adenovirus type 2 (reproduced from Horwitz, 1990b).

Early Region 1 (E1)

The E1A region of the adenovirus (1.3-4.5 map units) codes for more than six polypeptides ranging from 38-51 kDa, with the two main proteins being 289 and 243 amino acids long. The variety of resulting proteins arise from posttranslational modifications such as phosphorylation (reviewed in Horwitz, 1990b). The E1A gene is the first to be transcribed in productive infections, and its transcription does not depend on the action of any other viral gene products (Nevins *et al.*, 1979). The E1A proteins increase E1A gene transcription to high levels (Osbourne *et al.*, 1984), and also stimulate the promoter activities of other viral genes including the promoters for the E1B, E2A, E3 and E4 early genes as well as the major late promoter

(Winberg and Shenk, 1985). E1A proteins have also been found to stimulate transcription from non-viral promoters (reviewed in Berk, 1986).

The E1A region is critical in adenovirus transformation, and is required for the immortalization of cells in tissue culture (reviewed in Horwitz, 1990b). Complete transformation of rodent cells requires functions encoded in both E1A and E1B; however, transfection of cells with E1A alone can result in a cell with a partially transformed phenotype (Houweling *et al.*, 1980; Gallimore *et al.*, 1985). These cells have the potential to replicate indefinitely in media with serum, but do not display the morphological features or high saturation densities of cells transformed by both E1A and E1B (Berk, 1986). The domains in E1A required for transformation include the E1A N terminus and the two regions conserved between the various Ad serotypes, CR1 (Conserved region 1; amino acids 41-80) and CR2 (amino acids 121-139). These regions have been found to interact with a number of cellular proteins including the product of the retinoblastoma locus (Rb), p105. pRB and related proteins (p107 and p130) form complexes with the transcription factor E2F, that are disrupted by the binding of E1A to Rb. Release of the transcriptionally active E2F can stimulate cellular DNA synthesis in quiescent cells, and lead to cell immortilization (reviewed in Wong and Ziff, 1994). A third conserved region (CR3; amino acids 140-188) is also present in the E1A region, and it is essential for *trans*-activation of other virus transcriptional units (Kitchingman, 1993).

The E1A proteins are also responsible for both p53-dependent and p53-independent apoptosis. The 243-residue E1A protein causes an increase in the level of the cellular tumor suppressor gene p53, and induction of p53-dependent apoptosis. It is thought that the accumulation of p53 is induced by the induction of unscheduled DNA synthesis by E1A proteins, and that increased levels of p53 then activate cell death pathways (Querido *et al.*, 1997). In addition to these functions, the 289 and 243 amino acid proteins also induce sensitivity to tumor necrosis factor α (TNF-α). TNF-α initiates a programmed cell death response in

transformed cells which produces DNA fragmentation and cytolysis. Thus E1A is also capable of inducing p53-indepepndent apoptosis (White *et al.*, 1992).

The E1B region is located from 4.6 to 11.2 map units and codes for 2 major proteins of 19kDa and 55 kDa. In addition, E1B is predicted to code for at least three other polypeptides of 156, 93 and 84 residues (Takayesu *et al.*, 1994). This region of the Ad genome also plays an important role in transformation, specifically the 19 kDa and 55 kDa proteins. As previously stated, the E1A gene alone can immortalize rat cells (Houwelling *et al.*, 1980); however, complete, high efficiency transformation requires that the E1B gene be co-expressed with E1A (Barker and Berk, 1987). E1B gene products transform immortalized cells in a way that permits growth to high saturation densities, and allows for the formation of tumors in immunocompromised animals (Kitchingham, 1994). The expression of either E1B product allows E1A-dependent cell transformation to occur, although the expression of both proteins results in much higher transformation efficiencies (Querido *et al.*, 1997). The 19 kDa protein appears to protect against programmed cell death induced as a consequence of disruption of growth control pathways by E1A (Takayesu *et al.*, 1994). It appears that the 19kDa protein adopts a Bcl2-like role, thus inhibiting apoptosis and DNA degradation (Grand *et al.*, 1996). The E1B 55 kDa protein has been found to bind to the p53 tumor suppresser protein, which may help to control the cell cycle and transformation (Querido *et al.*, 1997). The 19 kDa protein also has *trans*-activating functions for E1A, E1B, E2, E3 and E4 as well as for the cellular heat shock protein promoter (reviewed in Horwitz, 1990b). Furthermore, the E1B 55 kDa protein has been found to interact with an early region 4 (E4) 25 kDa protein to allow efficient synthesis of viral DNA, to support expression of late viral genes, and to aid in host-cell shutoff (reviewed in Horwitz, 1990b).

Early Region 2 (E2)

The E2A region (67.9-61.5 map units) codes for a single-stranded DNA binding protein (DBP) which is heavily phosphorylated. This 72 kDa DBP is required for DNA replication both *in vivo* and *in vitro* (reviewed in Horwitz, 1990b). In addition, the DBP is also involved in other functions including repression of E4 transcription, mRNA stability (Babich and Nevins, 1981), assembly of virus particles (Nicholas *et al.*, 1983), transformation (Ginsberg *et al.*, 1974) and host range function (Klessig and Grodzicker, 1979). The E2B region codes for the precursor to the terminal protein (pTP), which is an 80 kDa polypeptide that is cleaved in the process of viral assembly to the covalently linked 55 kDa terminal protein (Chalberg and Kelly, 1981). The E2B region also codes for a 140 kDa Ad DNA polymerase, which plays an important role in DNA replication (reviewed in Horwitz, 1990b). The E2 promoter is transactivated by both the E1A protein, as well as the E4 6/7 protein (Swaminathan and Thimmapaya, 1996).

Early Region 3 (E3)

The E3 region is located at 76.6-82.6 map units, and about nine overlapping mRNAs are expressed by alternative processing of a common pre-mRNA that initiates from the E3 promoter (Chow *et al.*, 1979). The series of open reading frames in the E3 region code for proteins that modulate host functions in response to adenoviral infection (reviewed in Horwitz, 1990b). Of the six identified E3 proteins, the functions of four are known. The 19 kDa glycoprotein encoded by the E3 region has been shown to form a complex with the class I antigens of the MHC in the endoplasmic reticulum, thereby blocking the transport of the class I antigens to the cellular surface and thus protecting infected cells from lysis by cytotoxic T cells (Mahr and Gooding, 1999). The 14.7 kDa proteins and 10.4 kDa / 14.5 kDa (RIDα and β) proteins prevent lysis of adenovirus infected cells by TNF, counteracting the sensitivity to TNF cytolysis that the E1A gene products produce. The 10.4 kDa / 14.5 kDa proteins also stimulate the internalization

and processing of the receptor for epidermal growth factor, thus activating the cell and surrounding cells to become efficient factories for virus replication (Wold and Gooding, 1991).

Early Region 4 (E4)

The E4 region is located from 96.8-91.3 map units on the left strand. Primary transcripts from the E4 promoter are differentially spliced to give rise to at least 18 distinct mRNAs, which are predicted to encode for seven different polypeptides (Leppard, 1997). E4orf3 and E4orf6 play a role in the control of alternative splicing of the major late tripartite leader during lytic viral growth. The products of E4orf3 and E4orf6 appear to have redundant activities during Ad infection, and expression of either one is sufficient to support wild-type levels of virus production (Shtrichman and Kleinberger, 1998). E4orf6 is involved in host shutoff and transport of viral late mRNAs to the cytoplasm, following complex formation with the E1B 55 kDa polypeptide (Marcellus *et al.*, 1998). E4orf6 also binds to p53, to block p53-mediated transcriptional activation, to block p53-mediated apoptosis, and to enhance transformation by the E1 region (Shtrichman and Kleinberger, 1998). E4orf6/7 binds as a homodimer to transcription factor E2F to promote E2 promoter activity by ensuring correct spacing and orientation (Marcellus *et al.*, 1998). The E4orf1 gene encodes a transforming protein, while the E4orf4 protein regulates protein phosphorylation in the infected cell by binding to protein phosphatase (PP) 2A (Leppard, 1997). Little is known about the functions of the E4orf2 or E4orf3/4 proteins.

1.4.3 Late Gene Expression

As previously stated, the late phase of the adenoviral life cycle begins with the initiation of viral DNA replication. The switch from the early to late phases is a complex event that has not yet been fully explained; however, some evidence indicates that some of the controls are *cis*-

acting. There is no distinct division between the early and the late phases, as some late proteins are made early and some early proteins are made late. L2 and L3 transcripts have been found early, while the E2A mRNA and its polypeptide product the DBP are made both early and late (reviewed in Horwitz, 1990b). Synthesis of the late transcript begins at 16.45 map units and elongation occurs to approximately 99 map units. Transcription of the late region is under control of the major late promoter, and five major families of proteins (L1-L5) are produced through extensive splicing of the late transcript (reviewed in Lee *et al.*, 1995). Within each family the mRNAs overlap in a staggered fashion, sharing the same 3′ terminus but differing at their 5′ termini (Miller *et al*, 1980). Most of the late proteins are structural viral proteins or their precursors. Late region 1 encodes the 52, 55 kDa polypeptide pair, and polypeptide IIIa. Late region 2 codes for three proteins, polypeptides III, pVII and V, while late region 3 encodes for polypeptides II and pVI. Late region 4 encodes for the 100 kDa protein, the 33 kDa protein, and pVIII. Lastly, late region 5 encodes for the fiber protein, protein VI (Miller *et al.*, 1980). Two small RNAs are also synthesized from the adenoviral templates, but differ from mRNA in that they are synthesized by RNA polymerase III and they do not code for polypeptides. These virus-associated (VA) RNAs are 155 nucleotides long, and appear to control the rate of translation of the late polypeptides (reviewed in Horwitz, 1990b).

1.4.4 DNA Replication

Adenovirus DNA sequences begin to appear in large amounts at about 5 hours postinfection (Levy *et al.*, 1994). Ad DNA replication has been studied both *in vivo* and *in vitro*, including an *in vitro* system that can initiate and elongate exogenous adenovirus templates (Chalberg and Kelly, 1979). Ad5 possess an ITR that is 103 bp long and is located at either end of the genome. The ITR contains the replication origin of the viral DNA, and the most terminal

51 bp sequence of the ITR is sufficient for maximal initiation of DNA replication (Temperley and Hay, 1992). Thus the origin of replication is at the end of the genome, and either end of the molecule can be used for initiation (Lichy *et al.*, 1981). Ad DNA replication is semiconservative, and each strand elongates in a continuous 5′ → 3′ direction (reviewed in Horwitz, 1990b; Levy *et al.*, 1994). The replication has been proposed to occur in two separate ways, termed Type I replication and Type II replication (Figure 4). In Type I replication, strand elongation occurs from duplex DNA with strand displacement, in a modified version of rolling-circle replication. Initiation of this type of replication can begin at either end of the molecule; however, reinitiation will always occur at the same end as the first round. In Type II replication, replication occurs from a single-stranded template, likely the panhandle molecule that is formed from single stranded molecules of the Ad genome as a result of the terminal redundancies (Horwitz, 1990b).

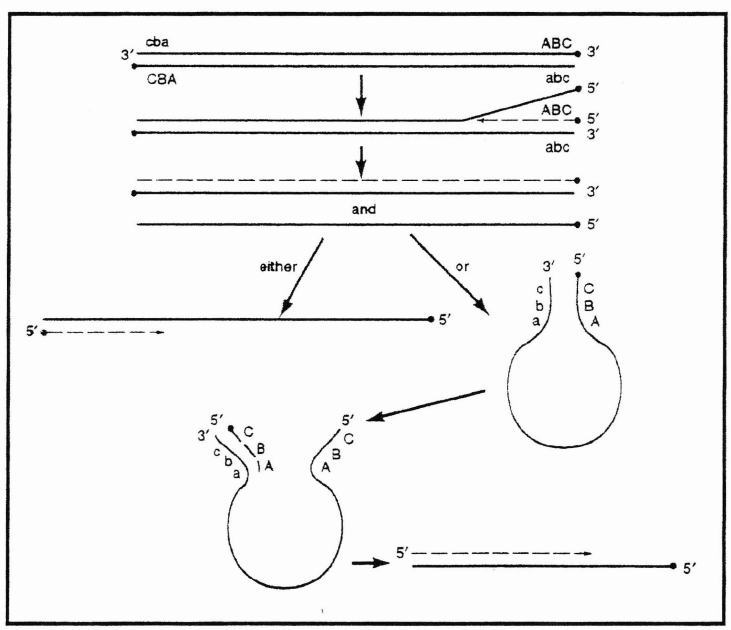


Figure 4. Proposed models for adenoviral DNA replication (reproduced from Levy, 1994)

Adenoviral DNA replication requires three virus coded polypeptides and four host proteins. The 80 kDa pTP is the primer for DNA replication (Lichy *et al.*, 1981). DNA replication is initiated by the first deoxycytidyl triphosphate (dCTP) becoming esterified via its 5′ phosphate group to the β-hydroxyl of a serine in the pTP. This pTP-CMP complex allows the free 3′-OH group in the cytidyl residue to be used as the primer for the growing DNA chain (Doerfler, 1994). The Ad DBP binds to the pTP and the terminal 18 bp of the Ad genome to complete the initiation complex (Yoshida *et al.*, 1995). During the initial phases of DNA replication, the 80 kDa pTP is cleaved to the 55 kDa TP (reviewed in Horwitz, 1990b; Doerfler, 1994). The 140 kDa DNA polymerase is the other viral polypeptide required for DNA replication (reviewed in Horwitz, 1990b; Levy *et al.*, 1994). The host protein nuclear factor I (NFI) is a polypeptide whose weight is between 47 and 160 kDa, and this protein binds to DNA sites near the origin of replication and is required for initiation and elongation. NFII is a 30 kDa complex of two polypeptides, which are 15 kDa and 17kDa. This is a type I topoisomerase that is required for full elongation late in the process of DNA replication. NFIII is a DNA binding protein that recognizes a sequence of the Ad genome 36-54 nucleotides from the origin of replication, and enhances activity of the origin. ORP A also acts near the origin of replication and binds within the first 12 nucleotides of the Ad genome, also assisting in replication (reviewed in Horwitz, 1990b).

1.4.5 Assembly of Virions

Viral assembly occurs in the nucleus of the infected cell, but begins in the cytoplasm. Initially, single polypeptides are assembled into capsomeres in the cytoplasm. The hexons are formed upon trimerization of hexon monomers, aided by a 100 kDa scaffold protein. The pentons are also formed, complete with the fiber attached. The hexon capsomeres then self-

assemble into empty structures which are known as the "light intermediate" capsids. This capsid contains a number of polypeptides, including 50 kDa and 39 kDa polypeptides, which act as scaffold proteins in the formation of the capsid (reviewed in Horwitz, 1990b). It has then been assumed that DNA enters the capsid through an opening at one of the vertices, after the capsid has been transported to the nucleus. Virions then accumulate in the nucleus, and are eventually released upon lysis of the cell (Levy *et al.*, 1994). Occasionally, some adenoviruses of group B are packaged defectively, leading to viruses which contain incomplete genomes which are referred to as defective particles (reviewed in Horwitz, 1990b).

1.5 GENE THERAPY

Gene therapy basically consists of the introduction of nucleic acid into the cells of a patient in order to use the expression of that nucleic acid for therapeutic benefit for the individual (reviewed in Morgan and Anderson, 1993; Smith, 1995). There are two different systems that can be used to introduce foreign genetic information into a patient. In the *ex vivo* method, target cells taken from a patient are genetically modified in a laboratory and then reintroduced into the patient to permit the *in vivo* expression of the gene of interest (reviewed in Brody and Crystal, 1994). In the *in vivo* method or the direct gene transfer method, the gene is directly delivered to the cell without the need for *ex vivo* manipulations. Direct gene transfer can be carried out by injection of naked plasmid DNA, liposome-encapsulated DNA, synthetic vectors linking foreign genetic material to ligands that specifically bind to cell surface receptors, or by using viral-based vectors that carry the gene of interest (reviewed in Haddada *et al.*, 1995). Initially, retroviruses were the viral vehicle of choice for gene therapy, due to the virus's ability to integrate its genetic material directly into the hosts chromosome resulting in a high efficiency of gene transfer into replicating cells (reviewed in Morgan and Anderson, 1993). However, there are a number of

disadvantages to retroviral vectors, including the difficulty involved with inserting large pieces of DNA into these vectors, the fact that most retroviruses are unable to infect non-dividing cells, and the risk associated with integration into the genome, including insertional mutagenesis (reviewed in Weatherall, 1995). Attention is now turning to other viruses, including adenoviruses, as gene delivery vehicles.

1.6 ADENOVIRUSES AS GENE THERAPY VECTORS

Adenoviruses have a number of advantages that make them attractive for use in gene therapy. First and foremost, adenoviral vectors are safe. Live oral adenovirus vaccines have been administered to over 10 million army recruits over the last 20 years with no detectable forms of toxicity encountered (Rubin and Rorke, 1988). Also, the adenoviruses that are most commonly used to make vectors are Ad2 and Ad5, and these strains do not cause tumors in rodents, and cause only mild respiratory infections in humans (reviewed in Kremer and Perricaudet, 1995). Furthermore, adenoviruses can accept large foreign DNA inserts; they can infect dividing as well as postmitotic cells; they can be concentrated to very high titers (reviewed in Haddada *et al.*, 1995); and they rarely integrate into the host genome, therefore having little chance to result in insertional mutagenesis (reviewed in Kremer and Perricaudet, 1995). Of the 400 current ongoing human gene therapy clinical trials, more than 27% utilize adenovirus vectors (AdVs; Wiley, Gene Therapy Website www.wiley.co.uk/genetherapy).

1.6.1 First-, Second-, and Third-Generation Adenovirus Vectors

As stated, adenoviral vectors are usually generated from the Ad2 and Ad5 strains, which have genomes of approximately 35 kbp. The maximum amount of DNA that can be packaged into the virions is approximately 105-106% of the wild-type genome, or about 2 kbp of extra

DNA (Ghosh-Choudhury *et al.*, 1987). In order to allow for larger inserts into the genome, it is necessary to delete viral DNA sequences, and then supply these lost gene functions in *trans* by a helper cell line. The most common deletions remove up to 3.2 kbp in the E1 region and over 2 kbp in the E3 region of the genome, leading to vectors which can accept inserts of up to 7 kbp (Graham and Prevec, 1991). These are referred to as first-generation Ad vectors. Because the E1 region is essential for replication of the virus, vectors with the E1 region deleted are termed replication deficient. These vectors must be propagated in 293 cells, a cell line transformed by Ad5 DNA which contains and expresses the left end of the genome (Graham *et al.*, 1977). The E3 region is nonessential for replication of adenoviruses in cultured cells, and thus these functions are not necessary to supply in *trans*. E1 deletions must not extend into the E1 region containing the coding sequence for protein IX, which is essential for packaging of the viral genome (Ghosh-Choudhury *et al.*, 1987). In order to allow for the insertion of larger genes, and to overcome some of the problems associated with first-generation Ad vectors such as transient gene expression and safety concerns (see section **1.6.5**), researchers have developed second and third-generation Ad vectors.

Second-generation Ad vectors involve deleting additional genes from the Ad genome. Generally, these vectors must be propagated and packaged in complementing cell lines that supply the missing gene functions in *trans*. Ad vectors containing deletions in the E1 and E3 regions, along with a deletion of the Ad polymerase (E2B) gene have been developed (Amalfitano *et al.*, 1998). Complementary cell lines have been produced to generate Ad vectors with deletions in the E1, E4 and pIX regions (Krougliak and Graham, 1995), or just the E1 and E4 regions alone (Wang *et al.*, 1995; Yeh *et al.*, 1996). Deletions have also been made in the E2A and E4 regions (Lusky *et al.*, 1998), and the preterminal protein has been deleted from some Ad vectors (Schaack *et al*, 1996). The rationale for these second-generation Ad vectors was that safety should be improved due to the further disabling of Ad replication, while

increasing the amount of space available for the insertion of foreign genes. Researchers are currently developing "gutless" vectors which contain only the inverted terminal repeats and packaging sequences around the transgene, with all the necessary viral genes being supplied in *trans* by a helper virus (Fisher *et al.*, 1996; Chen *et al.*, 1997; Schiedner *et al.*, 1998). These third-generation vectors can package 30 kbp of foreign DNA and contain no viral genes; however, their large-scale production remains a challenge (reviewed in Qualikene *et al.*, 2000).

1.6.2 Adenovirus Vectors and Cystic Fibrosis Gene Therapy

Adenoviral vectors have been extensively studied for their use in treating cystic fibrosis (CF). CF is a common, severe, autosomal, recessive genetic disease, which is caused by dysfunction of an epithelial cell surface cAMP (cyclic adenosine monophosphate) activated chloride channel. A rise in cAMP levels within the cells causes increased chloride secretion through the CFTR (cystic fibrosis transmembrane conductance regulator) channel into the lumen, resulting in a variety of effects for CF individuals including accumulation of mucus in the lungs and gastrointestinal tract (Colledge and Evans, 1995). Cystic fibrosis is a disease that is an attractive target for gene therapy because the gene required (CFTR) is well characterized and only low-level expression is required. Adenoviral vectors have a number of characteristics that make them suitable for CF gene therapy, including the ability of Ad vectors to accommodate the 4.5 kbp human CFTR cDNA, the natural tropism of Ad for respiratory epithelium (Crystal *et al.*, 1994), and the fact that they will efficiently infect non-replicating epithelial cells (Colledge and Evans, 1995). The majority of adenoviral vectors used for CF gene therapy have deletions in the E1 region, rendering them replication deficient. Initially, studies were done on nonhuman primates to study the effect of CFTR transfer to the lungs with adenoviral vectors (Engelhardt *et al.*, 1993). Initial reports of use with humans showed that the administration of AdCFTR to the

nasal and bronchial epithelium could correct the defective chloride channel for a short time (Zabner *et al.*, 1993; Crystal *et al*, 1994). However, high doses of the vector could result in adverse reactions including headache, fatigue, and fever, possibly due to release of interleukin-6 (IL-6), a cytokine known to induce these conditions (McElvany and Crystal, 1995). Improved Ad vectors to treat CF are continually being developed (Zuckerman *et al.*, 1999; Bigger and Coutelle, 2001; Perricone *et al.*, 2001).

1.6.3 Adenovirus Vectors and Cancer Gene Therapy

As more information about the causes of cancer is obtained, and the genes associated with cancer are discovered, the use of Ads for cancer gene therapy increases. While long-term expression of the genes of interest is essential for non-malignant therapies, such as that for cystic fibrosis, long-term expression is unnecessary for cancer gene therapy (Weitzmann *et al.*, 1996). Furthermore, a number of the vectors used in cancer gene therapy are replication competent, which is again different from gene therapy for other diseases. The E1 gene can be inserted into a first-generation virus under the control of a tumor-specific promoter, and theoretically, injection of this virus should allow for replication only in the tumor but not in the surrounding normal cells. This type of vector could be used to kill tumors directly by lysis, or by delivery of a 'suicide gene' such as the herpes-simplex-virus thymidine kinase gene (HSV-*tk*), which can kill infected and bystander cells following treatment with the pro-drug Gancyclovir (reviewed in Robbins *et al.*, 1998). A number of different groups of researchers are investigating the use of Ad vectors for suicide gene therapy of cancer (Wildner *et* al., 1999; Lee *et* al., 2001). A phase I clinical trial for the treatment of prostate cancer using a replication-deficient Ad vector containing the HSV-*tk* gene has been completed (Shalev *et al.*, 2000). Suicide gene therapy using Ad vectors appears to be very promising for the treatment of cancer (Freeman, 2000).

Adenovirus vectors can also be used to deliver tumor supressor genes in order to treat cancer (Wills *et al.*, 1994; Schuler *et al.*, 1998). Mutations of the tumor suppressor p53 are the most common genetic alterations observed in human cancer, and loss of this p53 function impairs cell cycle arrest as well as repair mechanisms involved in the response to DNA damage. By supplying the p53 gene, local control of the disease can be observed (Schuler *et al.*, 1998). A number of clinical trials involving Ad vectors and p53 genes are currently underway, including a trial for metastatic malignant liver tumours (Habib *et al.*, 1999), and a trial for the treatment of bladder cancer (Kuball *et al.*, 2002). Researchers continue to develop Ad vectors for the delivery of the p53 gene (Morrissey *et al.*, 2002). Furthermore, Ad vectors can be used to introduce genes that result in stimulation of an immune response to the tumor. This may include the delivery of a variety of cytokines, which have been shown to decrease tumor growth when ectopically expressed on tumor cells or in their microenvironment. Some success has been seen with the use of IL-2 for liver metastasis, and IL-12 for use in melanoma and breast cancer clinical trials (reviewed in Weitzman *et al.*, 1996).

1.6.4 Additional Applications for Adenovirus Vectors and Gene Therapy

In addition to their use in gene therapy for cystic fibrosis and cancer, adenoviral vectors are also used for a number of other gene therapy applications. Adenoviral vectors have recently been shown to be extremely efficient gene transfer vectors for liver-directed gene therapy (Raper *et al.*, 1998). Adenoviruses are being investigated for their potential use as gene vector systems to treat a number of neurodegenerative diseases. Ad vectors encoding for superoxide dismutase, tyrosine hydroxylase and GDNF are currently being investigated to treat Parkinson's disease, and vector systems are also being developed to treat motor neuron diseases (Barkats *et al*, 1998). Adenovirus vectors are also being investigated for the treatment of hemophilia A. Hemophilia A

is the most common inherited bleeding disorder, and is caused by a deficiency in coagulation factor VIII (Balague *et al.*, 2000). Replacement of the deficient factor with frequent intravenous injections of plasma concentrates or recombinant proteins is the standard treatment for the disease. Hemophilia is a good candidate for gene therapy treatment due to a number of reasons, including the fact that there is a simple and well defined cause-and-effect relationship between the protein deficiency and bleeding symptoms, and even 1% to 5% of the normal physiological levels of the protein are therapeutic (Zhang *et al.*, 1999). The liver is the site of factor VIII production, and upon intravenous injection the Ad vectors concentrate in the liver, making Ad vectors a promising system for the treatment of hemophilia A (Zhang *et al.*, 1999). Using a minimal adenovirus vector deleted of all viral sequences and containing the full-length human factor VIII cDNA, researchers have been able to demonstrate the sustained high-level expression of the factor and restoration of clotting activity in hemophilic mice (Balague *et al.*, 2000). These results show the promise of gene therapy for hemophilia, and support further investigation and clinical trials.

1.6.5 Problems with Adenovirus Vectors for Gene Therapy

1.6.5.1 Transient Gene Expression

There are a number of problems associated with adenovirus vectors. The first major drawback of the use of Ad vectors in gene therapy is that foreign gene expression is transient due to death of target cells resulting from host cellular immune responses. Expression of a first-generation adenovirus vector in the liver after intravenous administration can be extensive in the first 1-3 days, but after 3 weeks expression can decrease by >90% (Worgall *et al.*, 1997). It has been shown that cells carrying the recombinant viral genome express the transgene as desired; however, low level expression of viral genes also occurs. This results in the presentation of viral

proteins to the immune system, and the cells expressing these viral antigens are recognized by antigen-specific cytotoxic T cells and eliminated (Yang *et al.*, 1994; Kaplan *et al.*, 1997; Worgall *et al.*, 1997). Furthermore, the adenoviral infection can trigger a strong inflammatory reaction affecting the patient's health, and also leading to the shut down of transgene expression as a consequence of destruction of host cells (Barkats *et al.*, 1998).

In order to overcome these problems of transient gene expression, many researchers are attempting to construct improved vectors, which consist of greater deletions in the viral genome and thus have a decreased ability to express viral genes and less chance of being "recognized" by the immune system. These are the second-generation Ad vectors (Amalfitano *et al.*, 1996; Schaack *et al.*, 1996; Amalfitano *et al.*, 1998; Lusky *et al.*, 1998). It is the believed that deletion of viral genes necessary for Ad genome replication should block viral DNA replication and decrease viral protein production, resulting in a diminished immune response and extended duration of foreign gene expression (Amalfitano *et al.*, 1996). Using "gutless" vectors with no viral genes at all, Chen *et al.* (1997) have shown the stable expression of a transgene in mice muscles for up to 84 days, and Schieder *et al.* (1998) have shown expression of a transgene in mice for up to ten months. Recently, gutless Ad vectors have been shown to have improved transgene expression in the central nervous system of rats as compared to first-generation adenovirus vectors (Zou *et al.*, 2000), and gutless vectors expressing the human FVIII were shown to display increased duration and levels of expression when compared to an analogous early generation vector (Reddy *et al.*, 2002). However, some contradictory results have been reported showing that increased deletions in the adenoviral genome do not extend the *in vivo* persistence of the transduced cells and do not reduce the antivirus immune response in mice (Lusky *et al.*, 1998).

It has also been found that the expression of the E3 14.7K gene within the Ad vector can help to prolong gene expression. Harrod *et al.* (1998) found that a vector containing the E3

14.7K gene resulted in increased transgene expression in the respiratory epithelium of mice over a 21-day period as compared to an E1-E3-deleted Ad vector. The inclusion of the E3 region in an Ad vector used for arterial gene transfer was also found to reduce inflammation and prolong gene expression (Wen *et al.*, 2001).

1.6.5.2 Humoral Immunity

In addition to the problems of cell-mediated immune responses to adenoviral vectors, there is also the problem of humoral immunity which prevents adenovirus vector-mediated gene expression upon initial administration or re-administration of the vector. Adenovirus type 5 is commonly used for the construction of Ad vectors, and it also commonly infects humans. Thus many adults have pre-existing neutralizing antibodies and cytotoxic T lymphocytes (CTL) against adenoviruses (Schulick *et al.*, 1997). Injection of a recombinant adenovirus into these individuals would be expected to produce a strong secondary immune response, resulting in destruction of the cells (Ilan *et al.*, 1998). This is also the problem that is encountered when a repeat administration of an adenoviral vector is attempted, such as in the treatment of CF (Dong *et al.*, 1996; Kaplan *et al.*, 1996). Repeated administration of an Ad vector encoding the CFTR gene to patients with CF was shown to result in humoral immune responses (Zabner *et al.*, 1996).

A number of methods to overcome the problem with humoral immunity have been investigated. Because neutralizing antibodies directed against one adenovirus serotype do not block infection by another adenovirus serotype, one approach to achieving successful repeat administration is to alternate the serotype of the human adenovirus vector being used (Kovesdi *et al.*, 1997). It has also been shown that oral tolerization of rats to adenoviral antigens allows repeated administration of adenoviral vectors (Ilan *et al.*, 1998). Furthermore, it has been shown that the inclusion of the E3 region in adenoviral vectors allows long-term gene expression and

readministration in the liver (Ilan *et al.*, 1997). Because the E3 region contains many genes involved in the host immune response (gp19K, 14.7K), inclusion of this gene in vectors helps to inhibit the antiviral humoral immune response. Lastly, some success has been achieved in animal models through the use of immunosupressive drugs or monoclonal antibodies blocking the T cell receptor (reviewed in Rasmussen *et al.*, 1999).

Another method being used to overcome the problem of humoral immunity is to use adenoviral vectors that are constructed from serotypes other than human. These adenoviruses are attractive for their use in gene therapy because there is no pre-existing immunity to non-human Ads in humans; thus, there will be no strong secondary immune responses upon administration. One group of Ads that is being extensively studied is canine adenovirus type 2 (CAV-2) (Klonjkowski *et al.*, 1997). A CAV-2 E1-deleted vector was produced, and was shown to be able to direct expression of a transgene in human and non-human cells *in vitro* (Klonjkowski *et al.*, 1997). Development of CAV vectors for use in human gene therapy, as well as their characterization and comparison to human Ads continues (Kremer *et al.*, 2000). Bovine adenoviruses (BAVs) are also being investigated for their potential as gene therapy vectors. BAV 2 and 3 are currently the most extensively studied strains of bovine adenovirus, and the sequencing of the genomes has been completed, along with the identification of many key genes within the genomes (Salmon *et al.*, 1993; Salmon and Haj-Ahmad, 1994; Esford and Haj-Ahmad, 1994; Ojkic *et al*, 1997; Fitzgerald *et al.*, 1997; Yagubi *et al*, 1998). BAV3 has been shown to infect human cells without showing growth or cytotoxic effects, and viral gene expression was barely detectable (Rasmussen *et al.*, 1999). Recently, a replication-deficient chimpanzee adenovirus vector has been developed and tested in human cells (Farina *et al.*, 2001). Thus, a promising way to overcome the problem of humoral immunity in human gene therapy is to use adenoviral vectors based on serotypes other than human.

1.6.5.3 Tissue Tropism

Although adenovirus vectors are extremely attractive for their use in gene therapy, they tend to have promiscuous tissue tropism that leads to ectopic expression of the transgene. This lack of tissue tropism severely limits the applicability of Ad vectors in the gene therapy of many diseases. As previously stated, Ad tropism is determined by the interaction of the fiber and the receptors on the surface of the host cells. Unfortunately, the widespread distribution of the CAR receptor for Ad5 precludes the targeting of specific cell types (Douglas and Curiel, 1997). The lack of tropism of Ad vectors results in a decrease in the efficiency of gene therapy due to sequestration of the vector in non-target cells. In addition, the different receptors for the different Ad serotypes are often unevenly distributed. Adenovirus receptor density among tumour cell populations of the same histology has been hypothesized to influence transduction efficiency and therapeutic results in gene therapy strategies. A 5-fold variation in Ad receptor density was identified among head and neck squamous cell carcinoma cell lines (Li *et al.*, 1999).

One way to overcome these problems is by creating chimeric fibers, in order to redirect Ad vectors. A chimeric Ad5/Ad3 fiber allowed for an Ad vector to be retargeted to the Ad3 receptor, and resulted in an increase of gene transfer into ovarian cancer cells as opposed to a vector with an Ad5 fiber (Kanerva *et al.*, 2002). One group has looked into a number of approaches in order to modify the tropism of Ad vectors to accomplish targeted transduction of muscle cells for Duchene muscular dystrophy. These included incorporating targeting ligands into the adenovirus fiber, and creating chimeric Ad5/Ad3 fibers with altered receptor recognition profiles (Douglas and Curiel, 1997). Ad vectors have also been redirected to the pulmonary endothelium through the use of cationic liposomes and bispecific antibodies to endothelial cells (Ma *et al.*, 2002). Targeting has also been performed through the use of specific promoters that will only be expressed in certain tissues. This strategy proved successful in the expression of a

gene in the cardiac muscle under the control of cardiac-specific promoters (Griscelli *et al.*, 1998). Work is continuing in the area of Ad tropism and vector targeting to specific cells.

1.6.5.4 Replication Competent Adenoviruses

The last major problem associated with adenoviral vectors is the creation of replication competent adenoviruses (RCA) through recombination events (Kremer and Perricaudet, 1995). The presence of replication competent recombinant adenoviruses is a safety risk for gene therapy, because they can result in the mobilization and spread of the replication-defective vector viruses, and also in significant tissue damage and pathogenicity (Fallaux *et al.*, 1998). Occasionally, this generation of RCA is the result of E1-complementation in *trans* by wild-type adenovirus infections. This would be of particular concern for gene therapy involving cystic fibrosis due to the need for the recombinant adenovirus to be administered to the respiratory tract, the site that is naturally infected by wild-type adenoviruses. In an attempt to decrease the likelihood of this event, Imler *et al.* (1995) have produced a vector carrying the CFTR gene in which a mutation was introduced in the *cis*-acting sequences that control the encapsidation of the viral genome. The results of this mutation are that in the presence of the wild-type virus, the DNA containing the wild-type encapsidation sequences are preferentially packaged, and the recombinant virus will therefore be destroyed by the host immune response (Imler *et al.*, 1995).

A more common way in which the recombinant adenoviral vectors become replication competent is through recombination between the vector and the 293 packaging cell line (Lochmuller *et al.*, 1994; Hehir *et al.* 1996; Zhu *et al.*, 1999). In typical E1 deleted vectors, there are at least 450 bp of homologous sequences in the vector and 293 cells at the left-hand side of the transgene, and about 800 bp of homologous sequences at the right-hand side (Fallaux *et al.*, 1998). As a result of these homologous regions a dual recombination event can occur to the left

and the right of the transgene, resulting in the loss of the transgene and acquirement by the vector of the E1 region.

A number of different groups have used various approaches in order to overcome the problem of RCA generation. Hehir *et al.* (1996) modified their adenoviral vectors by deleting or rearranging the protein IX coding region, thereby greatly reducing the frequency of recombination between the vector and the 293 cells. In addition to altering the backbone of the vector to decrease the frequency of recombination, new helper cell lines can also be produced along with matching vectors. Fallaux *et al.* (1998) have developed helper cell lines which contain the Ad5 E1 region from bp 459-3510. They also developed matched Ad5 vectors, which lack nucleotides 459-3510, thereby removing any overlapping sequences of homology and thus preventing the generation of RCA by homologous recombination. A new cell line obtained by transfection of primary human animocytes with plasmid DNA expressing the E1A and E1B functions has also been produced. The generation of RCA is prevented in this cell line by designing the transforming plasmid to lack sequence overlap with current adenoviral vectors (Schiedner *et al.*, 2000). A HeLa-based E1-expressing cell line with no homology 5′ to E1 and reduced homology 3′ to E1 has been produced. This cell line has been shown to exclude the emergence of RCA for over 20 passages of an E1-deficient vector (Gao *et al.,* 2000). An additional HeLa-based cell line that expresses the E1 region of Ad5 has been developed, and was also shown to not generate RCA (Kim *et al.*, 2001).

1.6.5.5 The Death of Jesse Gelsinger

The problems with adenovirus vectors in gene therapy can best be exemplified by the tragic death of 18-year-old Jesse Gelsinger. Four days after receiving a very high dose (3.8 x 10^{13} virus particles) of an Ad vector carrying the ornithine-transcarbamylase (OTC) gene to

control his ammonia metabolism, Jesse Gelsinger died of multiple organ failure. He was the first patient in a gene therapy trial to die of the therapy itself (Marshall, 1999). To this day, the exact reason for Jesse's death is unclear. It was found that the Ad vector carrying the OTC gene invaded not just the intended organ (the liver), but also many other organs including the spleen, lung, thyroid, kidney, and brain. In spite of the massive dose of the vector that he received, only 1% of the transferred genes reached the target organ. This dissemination caused an activation of innate immunity, followed by a systemic inflammatory response that resulted in his death (Marshall, 1999). Recently, it has been suggested that perhaps the Ad virus used in the trial induced a forceful response from the complement system, as a result of pre-existing antibodies against adenoviruses. It has been proposed that a viral dose comparable to the level that Jesse received may raise the concentration of a key component of the complement system to a level that could start a damaging immune reaction (Bostanci, 2002). It is clear from this tragic event that there are still many areas of gene therapy that are not fully understood, and that perhaps the data acquired from animal trials can not always be directly applicable to human trials. However, it does appear that the death of Jesse Gelsinger was unusual. Since 1999, a number of documents have been compiled to assess the risk factors and safety of Ad vectors in human subjects, and the results have been promising (Crystal *et al.*, 2002; Harvey *et al.*, 2002).

1.7 ADENOVIRUSES AS VACCINES

Although the stimulation of an immune response by a transgene is an obstacle for gene therapy, it is exactly what is desired for vaccination. The purpose of a vaccine is to produce a comprehensive immune response (antibody-mediated, cell-mediated and mucosal), such that a subsequent infection with the virus will quickly be recognized and abolished. For vaccination, it is usually only necessary to express the transgene long enough to stimulate an effective immune

response (reviewed in Wilkinson and Borysiewicz, 1995). Furthermore, because the majority of viruses enter into their host via mucosal surfaces, the ideal vaccine should stimulate mucosal immunity. This type of immunity is best induced by intranasal or oral delivery, and thus the most effective vaccines would be designed for delivery by mucosal routes (Babiuk *et al.*, 1996).

Vaccines can be either live, inactivated or subunit vaccines (reviewed in Wilkinson and Borysiewicz, 1995). A new generation of viral vaccines is now being investigated, which involves the identification and isolation of the virulence factors of viruses and the proteins or glycoproteins involved in inducing neutralizing antibodies or cell mediated responses to the viruses (Babiuk *et al.*, 1996). Once these proteins and glycoproteins have been identified, they can then be placed into various expression systems, including viral vectors. Adenoviruses have great potential for their use as recombinant live viral vaccines for both animals and humans. First, live adenovirus vaccines for Ad4 and Ad7 prepared in enteric-coated tablets have been administered to millions of army recruits in the last 20 years and have proven to be safe (Rubin and Rorke, 1988). Furthermore, Ad5 infections are usually not associated with serious illness, and this is the strain from which most adenoviral vectors are constructed. Also, human adenoviruses naturally infect the epithelial cells of the respiratory tract, indicating their ability to induce mucosal immunity (Graham and Prevec, 1992). Thus, adenoviral vectors appear attractive for use in developing vaccines for humans and for animals.

The adenoviral vectors used to produce these recombinant vaccines can be either replication competent, or replication defective (reviewed in Wilkinson and Borysiewicz, 1995). Some replication-competent Ad5 vectors carrying HSV, VSV, or rabies glycoprotein-coding sequences in the E3 region have been shown to raise neutralizing antibodies in rhesus monkeys, cows, pigs, dogs, foxes, striped skunks, raccoons and mice (Graham and Prevec, 1992). Furthermore, since Ad vectors expressing genes from HPV, HIV, CMV and RSV generate immune responses in animals, they are currently being tested in humans (reviewed in Wilkinson

and Borysiewicz, 1995). Replication defective adenovirus vectors with deletions in the E1 region have been used to express a pseudorabies glycoprotein, a flavivirus nonstructural protein, EBV gp220/360 and the measles virus nucleocapsid, all resulting in an immune response in animals (reviewed in Wilkinson and Borysiewicz, 1995; Stephenson, 1998). A replication-deficient Ad vector expressing the envelope glycoprotein gene (*env*) of HIV-1 has been constructed, and was shown to induce both a humoral and CTL response in mice (Bruce *et al.*, 1999). Recently, an Ad5 vector was shown to be the most effective vaccine vector delivery system for HIV infections in humans, upon comparison to other potential vaccines (Shiver *et al.*, 2002). These results are promising for the development of recombinant adenoviral vaccines for man.

In addition to the use of adenovirus vectors for human vaccines, they are also being investigated for their use in developing vaccines for animals. Foot-and-mouth disease virus (FMDV) is an RNA virus which causes a highly contagious disease affecting cloven-hoofed animals including cattle, pigs and sheep. Vaccination plays a large role in the control of the disease, and recently a replication-defective Ad5 vector has been developed for use as a sub-unit vaccine against FMDV (Mayr *et al.*, 1999). This vector was found to induce the production of neutralizing antibodies in animals, and thus may be used for vaccination against the disease. Recombinant BAVs also have considerable potential to provide a protective mucosal immune response in animals, because BAVs naturally infect the respiratory and gastrointestinal tract of cattle (Mittal *et al.*, 1995). The first bovine adenovirus expression vector was generated in 1995 (Mittal *et al.*, 1995), and was based on BAV3. The vector was non-defective and contained a 696 bp deletion in the E3 region, into which the firefly luciferase gene was inserted. The vector was shown to infect cells and express luciferase, thereby proving that the BAV3 E3 gene products are not necessary for virus growth in cultured cells, and can therefore be replaced with foreign genes. Mittal *et al.* (1996) have also shown that this vector produces pulmonary lesions

in cotton rats similar to those produced by the wildtype BAV3 virus. This finding further verifies the idea that BAV vectors have excellent potential for the development of recombinant vaccines for cattle, and may also be suitable as vectors for human gene therapy. Recently, BAV-3 vectors expressing a bovine herpesvirus-1 glycoprotein were shown to induce protective immune responses in calves (Zakhartchouk *et al.*, 1999; Reddy *et al.*, 2000).

1.8 OBJECTIVES OF PROJECT

The most commonly used packaging cell line for replication deficient human adenoviruses is the 293 cell line (Graham *et al.*, 1977). However, this cell line has been shown to lead to the production of replication competent adenoviruses through the process of homologous recombination between the vector and the cell line. Thus the objective of this project was to produce a cassette that was used to modify the 293 cell line through the process of homologous recombination. The modification involved the removal of the first 380 bp of the adenoviral genome from the 293 cells, such that these sequences that serve as a region of homology on the left-hand side of the transgene in the vector would be removed from the cell line, and replication competent adenovirus formation will be prevented. In order to reach this objective, the following tasks were undertaken:

1) Identification and cloning of the left cellular-viral junction in 293 cells.
2) Engineering the plasmid that was used to modify 293 cells.
3) Transfection of 293 cells with the constructed plasmid, identification of the cells that had undergone homologous recombination, and characterization of the new cell line.

2. MATERIALS AND METHODS

2.1 BACTERIAL STRAIN

The bacterial strain used for cloning and propagation of plasmid DNA was *Escherichia coli* DH5α, with the genotype (F^-, ϕ80, δ*lac*Z, ΔM15, Δ(*lac*ZYA-*arg*F), U169, *deo*R, *rec*A1, *end*A1, *hsd*R17(rk^-, mk^+), *pho*A, *sup*E44, *thi*-1, λ^-,*gry*A96, *rel*A1) (Woodcock *et al.*, 1989). Bacterial cultures were grown in sterile Luria-Bertani broth (LB; 10% bacto-tryptone, 5% bacto-yeast extract, 10% NaCl, 4N NaOH to pH 7.0) overnight at 37°C. For storage, cells were frozen in 15% glycerol (3 ml of 50% glycerol added to 7 ml of cells), and stored at -80°C for long term maintenance. To propagate culture from frozen stocks, cells were thawed and streaked onto agar plates using a sterile inoculating loop. Single colonies could be isolated from these plates, and the plates could be stored short term at 4°C.

2.2 PREPARATION OF COMPETENT CELLS

Competent bacterial cells were prepared according to Sambrook *et al.* (1989). The *E. coli* was first streaked on agar plates to allow for the isolation of a single colony. Half of a colony was picked and inoculated into 2 ml of LB and the other half of the colony was inoculated into 2 ml of LB supplemented with 100 μg/ml ampicillin and grown overnight in a 37°C shaker bath. If the colony inoculated into LB with ampicillin showed no growth, then a 500 ml flask of LB was inoculated with 2 ml of overnight culture from the LB tube and grown to mid-log phase ($O.D._{550}$ = 0.45-0.55). The cells were then placed on ice and centrifuged for 10 min at 3500 rpm at 4°C in a Beckman Avanti J25 centrifuge using a JA-14 rotor. The supernatant was decanted, and the cells were resuspended in 20 ml of cold transformation buffer

(75 mM $CaCl_2$, 5 mM Tris, pH 7.6) and incubated overnight at 4°C. The next day the cells were centrifuged again at 3500 rpm for 10 min at 4°C, the supernatant was decanted, and the cells were resuspended in 4 ml of cold transformation buffer and incubated on ice for 45 minutes. To store competent cells for subsequent use, 50% glycerol was added to the cells (3 ml per 7 ml of cells), and 150 µl aliquots were placed into sterile 1.5 ml Eppendorf tubes and stored at -80°C for up to 3 months.

2.3 BACTERIAL TRANSFORMATIONS

Transformations were carried out as described by Sambrook *et al.* (1989). Competent cells were removed from storage in -80°C and placed on ice. Ligated DNA (usually 5 µl) was added to each tube, and the cells were incubated on ice for 30 minutes with gentle mixing of the contents every 10 minutes. The cells were then heat shocked at 42°C for 45 seconds, put back onto ice for 2 minutes, diluted with 850 µl of LB and grown for 45 minutes in a slow 37°C shaker. Following incubation, the cells were spread (150 µl per plate) on LB-agar plates supplemented with either ampicillin or kanamycin. For the transformation of plasmids that were to be plated on kanamycin plates, the incubation period in the 37°C shaker was extended to 90 minutes. The plates were then incubated at 37°C overnight in order to obtain colonies.

2.4 SMALL SCALE PLASMID ISOLATION

The method used for small scale plasmid isolation was based on the method of Sambrook *et al.* (1989). Individual colonies were first picked from plates using sterile wooden sticks and inoculated into 2 ml LB containing ampicillin. The cells were then grown at 37°C overnight, and the next day 1.5 ml of culture was transferred into a sterile Eppendorf tube. The cells were

then centrifuged for 30 seconds and the supernatant was aspirated. The cells were then resuspended in 200 µl of cell resuspension solution (50 mM Tris HCl (pH 7.5), 10 mM EDTA, 100 µg/ml RNase A) and vortexed well to resuspend the pellet. Next, 200 µl of cell lysis solution (0.2 M NaOH, 1% SDS) was added to each tube, and the tubes were inverted for 1 minute in order to mix the contents. Subsequently, 200 µl of neutralization solution (1.32 M K acetate, pH 4.8) was added to each tube and again mixed for 1 minute. The tubes were then centrifuged for 10 minutes at room temperature, and the supernatant was then transferred to a new sterile tube, being careful not to include any of the pellet. Isopropanol (300 µl) was then added to the tubes and mixed to allow for precipitation of the DNA. The tubes were again centrifuged for 10 minutes, and the supernatant was decanted and the tubes were air dried to remove any traces of alcohol. Lastly, the DNA pellet was resuspended in 100 µl of TE buffer.

2.5 LARGE SCALE PLASMID ISOLATION

Large scale plasmid extraction and isolation is essentially the same as described above, again being based on the methods of Sambrook *et al.* (1989). A pure colony was first picked from a plate and grown overnight in 2 ml of LB with ampicillin at 37°C. The following day, a 500 ml flask of LB with ampicillin was inoculated with 1-2 ml of the overnight culture and again allowed to grow overnight at 37°C. Next, the 500 ml overnight culture was pelleted by centrifugation at 5700 rpm for 10 minutes at 22-25°C in a Beckman Avanti J25 centrifuge using a JA-14 rotor. The supernatant was immediately decanted, and the cells were resuspended in 15 ml (per centrifuge tube) of cell resuspension solution. To aid in resuspension, the pellet was manually disrupted using the pipette. Next, 15 ml of cell lysis solution was added to each tube, and the contents were mixed gently by inversion until cell lysis was complete and the solution became clear and viscous. Following this, 15 ml of neutralization solution was added to each

tube, and the tubes were again mixed by inversion and then centrifuged at 9550 rpm for 15 minutes at 22-25°C. The supernatant was then filtered through sterilized coffee filters into clean centrifuge bottles, and then 22 ml of room temperature isopropanol was added to the supernatant and mixed by inversion. The tubes were then centrifuged at 9550 rpm for 15 minutes at 22-25°C, the supernatant discarded and the DNA pellet was air dried at room temperature. Lastly, the DNA was resuspended in 2 ml of TE buffer and aliquoted by 1 ml into 2 sterile Eppendorf tubes.

2.6 CESIUM CHLORIDE GRADIENT CENTRIFUGATION

Ethidium bromide - cesium chloride gradient centrifugation (Sambrook *et al.*, 1989) was used to prepare high grade plasmid DNA for purposes such as transfection into mammalian cells. Cesium chloride was added to DNA suspended in TE buffer, such that the final density of the solution was 1.57 – 1.59 g/ml. Subsequently, 500 µl of ethidium bromide (10 mg/ml) was added to the DNA, and the solution was transferred to Beckman Optiseal ultracentrifuge tubes. The tubes were then centrifuged in a Beckman Optima XL-100K ultracentrifuge in a NVT65 rotor for 22 hours at 65,000 rpm and 22°C. After centrifugation, the red band containing the supercoiled plasmid DNA was removed with a needle and syringe, and extracted several times with isoamyl alcohol (saturated with CsCl and distilled water), to remove the EtBr.

DNA samples purified using the cesium chloride gradient technique were dialysed prior to use in mammalian cell culture transfection. A 5 cm piece of Spectra/Por® 7 (Spectrum Laboratories, Inc.) dialysis membrane tubing was boiled in distilled water for 15 minutes prior to use. After the tubing had cooled, one end was clamped and the DNA was transferred into the tube and then the other end was clamped. The dialysis tubing containing the DNA was then placed into a 2L flask containing 0.1X SSC buffer (15 mM NaCl, 1.5mM $Na_3C_6H_5O_7.2H_2O$) and

stirred at 4°C for 1 hour. The 0.1X SSC buffer was then changed, and the tubing and DNA were stirred overnight at 4°C. The following morning the DNA was transferred to an Eppendorf tube and the concentration determined.

2.7 DNA CONCENTRATION DETERMINATION

The DNA concentration was determined by measuring the absorbance at 260 nm (Hitachi U-2000 Spectrophotometer) and applying the following formula :

$$[DNA] = (A_{260} \times \text{dilution factor} \times 50) / 1000 \ \mu g/\mu l$$

The purity of DNA was determined by measuring the absorbance of the sample at 260 nm and 280 nm. An OD_{260}/OD_{280} of 1.8 to 2.0 was used to indicate a pure preparation.

2.8 DNA SEQUENCING

DNA sequencing was performed by Norgen Biotek Corp. (St. Catharines, ON), using a Visible Genetics Inc. Long-Read Tower. Sequence alignments and comparisons were performed using the BLAST software (Altschul *et al.*, 1997), available through the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov).

2.9 RESTRICTION ENZYME DIGESTIONS

Restriction enzymes were used as specified by the manufacturer (New England Biolabs). Typical reaction mixtures were 20 µl in total volume and consisted of 1-2 µg of DNA, 2 µl of recommended 10X restriction enzyme buffer, 2µl of BSA for a final concentration of 0.01 µg/µl if required, and 1-10 units of enzyme. One unit of enzyme is defined as the amount of enzyme required to digest 1µg of λ DNA in one hour at 37°C in a total reaction volume of 50 µl. The

total volume was then brought up to 20 µl with distilled, autoclaved water. Digestions were generally left overnight at 37°C.

2.10 SHRIMP ALKALINE PHOSPHATASE

In order to prevent self-ligation and recirculization of plasmid DNA in a reaction mixture, Shrimp Alkaline Phosphatase (SAP) (Boehringer Mannheim) was frequently used to remove the 5′ phosphate groups from one of the DNA digestions. Generally, 30 µl of digested DNA mixture, 6 µl of the 10X SAP buffer (0.5 M Tris-HCl, 100 mM $MgCl_2$, pH 8.5), and 2 µl of the SAP enzyme were brought up to a total volume of 60 µl with distilled water and incubated at 37°C for 1 hour.

2.11 KLENOW TREATMENT OF DNA

Klenow (NEB) was used according to the manufacturer's recommendations to fill in 5' overhangs generated by some restriction enzymes, in order to generate blunt ends. The Klenow fragment is the large proteolytic product of *E. coli.* DNA Polymerase I, which retains polymerization and 3' → 5' exonuclease activity but has lost 5'→ 3' exonuclease activity. A typical reaction mixture contained 0.5 – 1 µg of DNA, 4 µl of 10X EcoPol buffer (10 mM Tris-HCl, 5 mM $MgCl_2$, 7.5 mM DTT, pH 7.5), 33 µM of each dNTP, and one unit of Klenow per µg of DNA in a 40 µl total reaction volume. The reaction was carried out at room temperature for 15 minutes and then phenol extracted to remove the Klenow and unreacted dNTPs.

2.12 ENZYME REMOVAL AND EXTRACTION

2.12.1 Phenol Extraction

Prior to ligation or any additional steps involving enzymes, the enzymes used in the digestion or dephosphorylation had to be removed. One method used for this was phenol extraction (Sambrook *et al.*, 1989). The volume of the digestion mixture was first brought up to 100 µl by adding distilled water. Next, 200 µl of cold phenol:chloroform:isoamyl alcohol (25:24:1) (redistilled phenol, chloroform, isoamyl alcohol, 0.1% 8-hydroxyquinoline (w/v), saturated in TE buffer (pH 8) and stored at 4°C) was added to the mixture. The suspension was mixed gently by inversion for 2-3 minutes and then centrifuged for 5 minutes at 1200 rpm in a Sorvall MC 12V benchtop centrifuge to separate the phases. The top aqueous layer was transferred to a new Eppendorf tube, being careful not to include any of the organic layer. Ten µl of 3.5 M sodium acetate (pH 7.5) was then added, followed by 220 µl of cold 95% ethanol and an additional 5 minutes of centrifugation in order to precipitate the DNA. The supernatant was then decanted, and the DNA was washed in 200 µl of cold 70% ethanol. The tube was vortexed to ensure that the entire pellet was washed, and was then centrifuged again for 5 minutes. The recovered pellet was air dried at room temperature and then resuspended in 20-100 µl of distilled water or TE buffer (100mM Tris, 1 mM EDTA; pH 7.8).

2.12.2 Gene Clean From Agarose Gel

Individual bands of DNA can be excised and purified directly from the agarose gel using the Bio 101 GeneClean® kit. The GeneClean® kit removes salts, RNA, proteins, EtBr and enzymes from the DNA (Bio 101). In order to excise bands directly from the gel, thicker 0.9% agarose gels were made to separate the fragments. A sharp, clean razor blade was used to cut the

appropriate band from the gel under low UV light, and the band was placed into a previously weighed Eppendorf tube. The agarose was weighed, and 3 volumes of 6 M NaI stock solution was added to the Eppendorf tube. The tube was then incubated at 45-55°C for 5 minutes, mixing occasionally until the gel slice was completely dissolved. Five µl of Glassmilk® (a suspension of silica matrix in water) was then added to the DNA, and the tube was placed on ice and the contents mixed every 1 to 2 minutes for a total of 5 minutes. The suspension was then centrifuged for 5 seconds and the supernatant removed. The pellet was then resuspended in 10 to 50 volumes (usually 300 µl) of ice cold NEW™ Wash, using the pipette tip to manually disrupt the pellet. The DNA was again centrifuged and the supernatant removed, and the wash was repeated two more times. After the last wash the pellet was completely dried, resuspended in 30 µl of TE buffer or distilled water and incubated at 45-55°C for 2 or 3 minutes to elute the DNA. Lastly, the suspension was centrifuged for 30 seconds to make a pellet, and 20µl of the supernatant containing the DNA was carefully collected.

2.12.3 Gene Clean From Solution

The Geneclean® kit (Bio 101) can also be used to purify DNA that is in solution, in order to remove restriction and modifying enzymes, and to clean up a reaction mixture. The protocol is exactly the same as above, however no initial incubation at 45-55°C is required because there is no agarose to dissolve.

2.12.4 QuickSilver™

DNA could also be purified both from agarose gels and in solution using the QuickSilver™ kit (Norgen Biotek Corp.). The procedure for purifying DNA using the

QuickSilver™ kit is virtually the same as the Geneclean® kit, except that Norgen Binding Resin is used instead of Glassmilk®. Generally, 10µl of resin was used to purify DNA, and the final elution step was carried out at 37°C instead of 45-55°C.

2.12.5 Heat Inactivation

Some enzymes used could be inactivated simply by heating at 65°C for 20 minutes. In order to determine if an enzyme could be heat inactivated, the suppliers catalogue (NEB) was consulted. For heat resistant enzymes, one of the previously described methods was used.

2.13 LIGATIONS

DNA ligations were carried out using T4 DNA ligase (MBI), which catalyzed the formation of a phosphodiester bond between adjacent nucleotides with a 5' phosphate group and a 3' hydroxyl group. Ligations were usually left overnight at 16°C, or for 1-3 hours at room temperature. Occasionally, ligations were left overnight at room temperature. Ligation mixtures usually consisted of 1-2 µg of vector DNA, and suitable amounts of insert DNA to give 1:1, 1:2 or 1:3 ratios of vector DNA: insert DNA. Also, 1- 10 units of T4 DNA ligase were used, and a volume of 10X ligase buffer (400 mM Tris-HCl, 100 mM $MgCl_2$, 100 mM DTT, 5 mM ATP, pH 7.8) that was equal to 10% of the total volume. For blunt end ligations, 5% PEG 4000 (50% w/v) was also added. The final volume was obtained by adding distilled water.

2.14 PREPARATION AND LIGATION OF LINKERS

The linkers used for cloning and modification of restriction sites were made from oligonucleotides (Norgen Biotek Corp.) such that they are dissolved at a final concentration of 1

pmole/µl. The appropriate amounts of each oligonucleotide required to form a DNA duplex were mixed in distilled water in an Eppendorf tube, and then heated at 90°C for 5 minutes. The linkers were then cooled to room temperature in order for annealing to occur, before being used in subsequent ligations. The ligations were then set up such that 3.5 pmoles of double-stranded linker were ligated to 0.5 µg of linearized DNA in a total volume of 20 µl (D. Bautista, Ph.D. thesis, McMaster University, Hamilton, Ontaio, Canada, 1984).

2.15 MOLECULAR MODIFICATION OF RESTRICTION ENZYME SITES

Occasionally, it was necessary to eliminate a restriction enzyme site that was present in a plasmid. Usually this involved a plasmid containing two recognition sites for a particular restriction enzyme, and one site would be destroyed such that the other site then became a unique site. The first step involved partially digesting the plasmid with the restriction enzyme, and was based on the methods of Parker *et al.* (1977). In a typical reaction, 20 µg of DNA, 10 µl of appropriate 10X restriction enzyme buffer, 1 unit of restriction enzyme and 2.6 µg/ml of ethidium bromide were added together and brought up to a final volume of 100 µl. The incubation time that resulted in the largest amount of plasmid DNA being cut at only one of the restriction enzyme sites was determined empirically. For example, 10 µl aliquots were removed from the reaction every 5 minutes and the enzymatic reaction was stopped by adding 5 µl of 6X loading buffer. The samples were then run on an agarose gel to determine the incubation time that resulted in the greatest amount of linear DNA.

Once the most desirable incubation time was determined, the reaction was run again and stopped at this time by the addition of 6X loading buffer. The digest was then run in a thick well on an agarose gel, and the band excised and purified using the Geneclean® kit (Bio 101). The purified, linear DNA would then be treated with Klenow and subsequently ligated together,

resulting in the destruction of the restriction enzyme site. The plasmids then needed to be screened to determine which of the two restriction sites had been destroyed.

2.16 AGAROSE GEL ELECTROPHORESIS

After digestion of the DNA, the fragments could be separated and visualized using agarose gel electrophoresis. Agarose gels were made by dissolving 0.9% w/v agarose in TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.0), allowing the gel to solidify in a tray with an appropriately sized comb, and then storing the gel in TAE buffer with 0.15 μg/ml ethidium bromide (EtBr). Generally, 5 μl of loading buffer (0.05% bromophenol blue, 20% glycerol, 2%SDS) was added to the DNA samples, and about 20 μl of this was loaded into a well. N1 DNA Ruler (Norgen Biotek Corp.) or PCR-Sizer (Norgen Biotek Corp.) molecular weight markers were also loaded on to the gel to help in the estimation of fragment sizes. DNA fragments were separated at 1-10 V/cm, and then the gel was viewed under UV light using the Bio-Rad Gel Doc 1000 system, and photographed using the Multi-Analyst Version 1.1 software (Bio-Rad).

2.17 POLYMERASE CHAIN REACTION (PCR)

2.17.1 Primers and Reaction Conditions for PCR

Primers used in PCR experiments were designed using VECTOR NTI 4.0 software (InforMax) and synthesized by Norgen Biotek Corp. (St. Catharines, ON). The primers were typically designed to be 20 to 26 nucleotides in length, possess a GC content of more than 50%, and have similar melting temperatures for primer pairs in the range of 56-65°C.

A typical PCR reaction was carried out in 50 µl volume and contained template DNA, 1-2 units of *Taq* polymerase (MBI Fermentas), 5 µl of 10X buffer (100mM Tris-HCl (pH8.8), 500 mM KCl, 0.8% Nonidet® P40), 0.2 mM of dNTP mix (MBI Fermentas, equal amounts of dATP, dTTP, dGTP, dCTP), 0.5 uM of forward and reverse primer, and 0.5-2.5 mM of $MgCl_2$. The PCR reactions were run in a SingleBlock™ System (Easycycler™ Series, ERICOMP INC.), with mineral oil being placed over the samples in order to prevent evaporation. Samples were initially denatured for 3 to 5 minutes at 94°C. Occasionally hot-start PCR was performed, and the *Taq* polymerase and buffer would be added at this point, following the initial denaturation but prior to the beginning of the cycling. The template DNA was typically amplified in 35 PCR cycles consisting of a 1 minute denaturation at 94° C, annealing for 1 minute at 52-60°C (depending on the primer pair), and extension for 1-2 minutes at 72°C. Following the completion of the 35 PCR cycles, a final extension was carried out for 15 minutes at 72°C. For specific PCR primers and reaction conditions, please see the results section.

2.17.2 Expand™ High Fidelity PCR System

For PCR reactions that required a high degree of fidelity, the Expand™ High Fidelity PCR System was used (Boehringer Mannheim). The system is composed of a unique enzyme mix containing thermostable *Taq* DNA and *Pwo* DNA polymerases, and is designed to give PCR products with high yield, high fidelity, and high specificity. The PCR reaction volume was again 50 µl, and contained template DNA, 2.6 units of enzyme mix, 5 µl of 10X buffer (20 mM Tris-HCl, pH 7.5, 100 mM KCl, 1 mM dithiothreitol, 0.1 mM EDTA, 0.5% Tween® 20, 0.5% Nonidet® P40, 50% glycerol) supplemented with 15mM $MgCl_2$, 0.2 mM dNTP mix, and 0.5 uM

of each primer. The reactions were run in the same machine and using the same programs as described above.

2.18 MAMMALIAN CELL LINES

The 293 cell line is a human embryonic kidney cell line that was derived by transforming human cells with mechanically sheared human adenovirus type 5 DNA (Graham *et al.*, 1977). The cells contain and express the E1 region of Ad5, and thus are used as a packaging cell line for E1 defective adenovirus vectors.

Cells were grown in Minimum Essential Medium (MEM) supplemented with 2 mM GlutaMAX-1 (Gibco BRL), 0.225% w/v sodium bicarbonate, 10% fetal bovine or horse serum, and 1% antibiotic/antimycotic [10,000 units/ml penicillin, 10,000 µg/ml streptomycin sulfate and 25 µg/ml amphotericin B (Gibco BRL)].

Cells were typically passaged twice a week, when 80-90% confluency was reached. The medium was removed by aspiration and the cells were washed with 5 ml of 1X saline citrate (134 mM KCl, 15mM $Na_3C_6H_5O_7.2H_2O$). Subsequently, 3 ml of 1X saline citrate was added to the 150 mm dish and incubated at 37°C until the cells in culture partially detached from the bottom of the dish. After tapping the side of the dish to assist in complete detachment, the cells were resuspended in fresh media and split between new dishes in a 1:2 or 1:3 ratio, noting the passage number on the dish. The cells were maintained in a humid CO_2 incubator (Fisher Scientific IsoTemp® incubator; Model 546), which was kept at 37°C and at a 5% CO_2 level.

2.19 CELL FREEZING AND THAWING

Cell freezing was carried out as outlined by Freshney (2000). Cells were grown to late log phase, lifted off the plate using saline citrate, and then centrifuged at 600 rpm for 3 minutes at room temperature (IEC Centra-8R Centrifuge). The media was aspirated and the cells then resuspended in 4 ml of fetal bovine serum containing 10% dimethyl sulfoxide (DMSO). The cells were then dispensed into pre-labeled 2ml cryogenic vials (Nalgene). In order to freeze the cells slowly, the vials were placed on cotton wool in a foam box and placed at -80°C overnight, after which they were transferred to liquid nitrogen for long term storage.

To recover frozen cell stocks, cells in cryogenic vials were thawed by heating quickly in a 37°C water bath. Once thawed, the cells were poured into a 150 mm culture plate containing media supplemented with 10% serum, and swirled well to mix. As soon as the cells attached to the plate, the media was changed in order to remove the toxic DMSO.

2.20 CALCIUM PHOSPHATE TRANSFECTION

The calcium phosphate procedure used to transfect the 293 cells in this study was based on the method developed by Graham and van der Eb (1973). The day prior to transfection, cells were subcultured and plated at a density of 3×10^5 or 5×10^5 cells per 35mm plate. Three hours before transfection, the media on the cells was changed to media supplemented with 10% fetal bovine serum. All buffers and solutions were warmed to room temperature prior to transfection. For each 35 mm plate, either 2 µg or 5µg of DNA was mixed in TE buffer (pH 8.0) to a final volume of 90 µl, to which 10 µl of 2.5M $CaCl_2$ (filter sterilized) was added. The DNA- $CaCl_2$ mixture was slowly added to 100 µl of 2X HEPES (280 mM NaCl, 10 mM KCl, 1.5 mM $Na_2HPO_4.2H_2O$, 12 mM dextrose, 50 mM HEPES, pH 7.05, filter sterilized) with constant mixing. Once combined, the mixture was incubated at room temperature for 1 minute to allow

for the formation of calcium phosphate – DNA complexes. After the incubation period, the solution was briefly vortexed and then added dropwise to the cells with constant swirling. The cells were then incubated at 37°C, and the media changed after 4 hours. Cells were then allowed to grow for 48 hours, after which time selection began.

2.21 SELECTION WITH GENETICIN

Cultured cells differ widely in their sensitivity to Geneticin (G418; Gibco BRL). The appropriate concentration to use for selecting stably transfected cells had to be determined experimentally. Non-transfected 293 cells were subjected to varying levels of G418 during a 2-week period to determine the lowest level that would bring about total cell death in that period. From this it was determined that 150 – 200μg/ml of G418 would be the ideal concentration to use to select stably transfected 293 cells. Thus, 48 hours following transfection, new media containing G418 was added to the cells to select for those expressing the neomycin resistance gene. Fresh growth media containing G418 was added every 4 days, and selection was maintained for 2 weeks or until all the control cells were killed.

2.22 SELECTION WITH GANCYCLOVIR

Once stably transfected cells had been selected for their resistance to G418, cells which had presumably undergone homologous recombination were selected for using Gancyclovir (GANC, Sigma). GANC is a pro-drug nucleoside analog that, upon expression of a viral gene encoding thymidine kinase, is converted to its phosphorylated active analog which is incorporated into the DNA of replicating eukaryotic cells, resulting in cell death. The level of GANC used for selection (2×10^{-6} M) was based on literature values (Mansour *et al.*, 1988). To

ensure that 293 cells were not sensitive to GANC, cells were grown for 2 weeks in media supplemented with 2 x 10^{-6} M GANC, and no cell death occurred.

Selection with GANC began after the 2 week selection in G418. Cells were fed with media that contained 150 µg/ml G418 and 2 x 10^{-6} M GANC, and again fresh growth media containing the selective agents was added to the plates every 3 to 4 days. Selection was maintained for 12 days, after which time $G418^r$ and $GANC^r$ colonies could be picked and expanded.

2.23 ISOLATION OF $G418^r$ AND $GANC^r$ CELLS

2.23.1 Isolation of $G418^r$ and $GANC^r$ Pools

Following the selection in G418 and GANC, several foci were observed to be continually growing. In order to isolate these foci of interest, pools of cells were selected from the 35mm dishes through the use of sterile aluminum cloning rings, as described by Freshney (2000). The foci to be picked were marked on the underside of the dish using a marker. The media was then removed from the dish, and the cells washed with 1 ml of PBS (140 mM NaCl, 2.6 mM KCl, 4 mM Na_2HPO_4, 1.5 mM KH_2PO_4; pH 7.4). Sterile forceps were then used to dip a cloning ring in silicone grease (DOW Corning), and then the ring was placed around the desired colonies. This was repeated for each desired pool in the dish. Following the placement of the rings, each ring was filled with a few drops of saline citrate, which was then aspirated in order to wash the cells. The ring was again filled with saline citrate, and the plate was then placed into the 37°C incubator for 3-5 minutes or until the cells were detached. Once the cells had lifted off the plate, media was added to each of the rings and the cells were then transferred to a 20mm plate for expansion.

2.23.2 Isolation of Single G418^r and GANC^r Foci

Once a promising pool had been identified, single homogeneous foci had to be isolated from the pools. Media was first aspirated from the well that contained the promising pool, and the cells were washed twice with 1 ml of PBS. Following washing, the cells were detached from the plate using a few drops of saline citrate and incubated at 37°C for 10 minutes to ensure that the cells were singly suspended. Two ml of media was then added to the cells, and the cells were counted using a hemocytometer. Media was then added such that the final concentration of cells was 2 cells/ml of media. Then, 25 ml of media was transferred to a 150mm plate, and the plates returned to the incubator for growth. Media was changed after 1 week, and after 2 weeks single, isolated foci could be found. Various foci of different size and morphology were then picked and transferred to 20mm plates using the cloning ring procedure described above.

2.24 HISTOCHEMICAL ASSAY FOR lacZ ACTIVITY

Cells previously transfected with a plasmid carrying the *lacZ* reporter gene were stained for *lacZ* activity. The media was first aspirated from the cells, and then the cells were washed with 1 ml of PBS. The cells were then overlaid with 1 ml of 4% paraformaldehyde (pH 7.2) and incubated at 37°C for 2 minutes. The paraformaldehyde was then aspirated and the cells were washed 2 times with 1 ml of PBS. The cells were then overlaid with 2 ml X-gal stain (35 mM $K_3Fe(CN)_6$, 35 mM $K_4Fe(CN)_6$, 1 mM $MgCl_2$, in PBS; pH 7.4, supplemented with 1 mg/ml of X-gal) and incubated at 37°C overnight. The following day, the number of yellow and blue cells could be counted and transfection efficiency determined.

2.25 GENOMIC DNA ISOLATION

Genomic DNA was isolated from cells in the 20mm plates in order to screen for homologous recombination (Sambrook *et al.*, 1989). Media was first aspirated from the plates, and the cells were then washed with 500 µl of PBS (140 mM NaCl, 2.6 mM KCl, 4 mM Na_2HPO_4, and 1.5 mM KH_2PO_4, pH 7.4). Five hundred µl of Pronase-SDS lysis buffer (0.01M Tris-Cl, 0.01M EDTA, 0.5% SDS, 1 mg/ml Pronase; pH7.8) was then added to the cells and swirled to mix, and the plate was incubated at 37°C for 2 hours. The cell lysate was then collected into Eppendorf tubes, and 500 µl of cold phenol:chloroform:isoamyl alcohol (25:24:1) (redistilled phenol, chloroform, isoamyl alcohol 0.1% 8-hydroxyquinoline (w/v), saturated in TE buffer (pH 8) and stored at 4°C) was added and the contents inverted gently for 10 minutes. Following mixing, the suspension was centrifuged for 5 minutes at 1200 rpm in a Sorvall MC 12V benchtop centrifuge. The top aqueous layer was then removed and transferred to a new Eppendorf tube, and an equal volume of water-saturated chlorofom was added. The tubes were then inverted gently for 10 minutes to remove any traces of phenol. Again the suspension was centrifuged for 5 minutes, and the upper aqueous layer transferred to a new Eppendorf tube. Ten percent 3.5 M sodium acetate (pH 7.5) was then added, followed by the addition of 2 volumes of ice cold 95% ethanol with mixing by inversion until high molecular weight DNA appeared. The tube was then centrifuged for an additional 3 minutes, the ethanol decanted and the DNA pellet washed in 200 µl of ice cold 70% ethanol. Finally, the tube was again centrifuged for 3 minutes, the supernatant decanted and the DNA pellet resuspended in 100 µl of MilliQ water and the concentration determined.

3. RESULTS

The objective of this project is to modify the 293 packaging cell line (Graham *et al.*, 1977) such that replication competent adenovirus formation can be eliminated during recombinant adenovirus propogation. Currently, 293 cells are used as a packaging cell line for E1-deficient Ad vectors, because the cells are able to provide the E1 gene products in *trans* due to constitutive expression of the Ad5 E1 region. The 293 cells contain the Ad5 genome from nucleotide 1 to nucleotide 4344 (Louis *et al.*, 1997), and typical E1 replacement vectors contain homologous sequences on both the left-hand side and right-hand side of the transgene. The result of this homology is that replication competent adenoviruses can be generated through homologous recombination between the vector and the packaging cell line, with crossover events occurring on both sides of the transgene (Figure 5). In this project, the goal is to circumvent the problem of wild-type reversion by modifying the 293 cells in such a way that the regions of homology on the left-hand side of the transgene are removed from the cells, and thus a double crossover event is prevented from occurring (Figure 5).

In order to modify the 293 cells, a strategy involving homologous recombination was investigated. A targeting cassette was designed and constructed, which upon integration into the 293 genome via homologous recombination would result in the removal of the first 380 bp of the adenoviral genome. Considering that non-homologous recombination is 1000 times more likely to occur than homologous recombination (Capecchi, 1990), a number of key features were incorporated into the constructed cassette so that the chances of generating homologous recombinants would increase. First, the left cellular-viral junction of 293 cells had to be included in the cassette to serve as the left-end region of homology. Secondly, a dominant selectable marker needed to be present in order to select for the cells that had taken up the

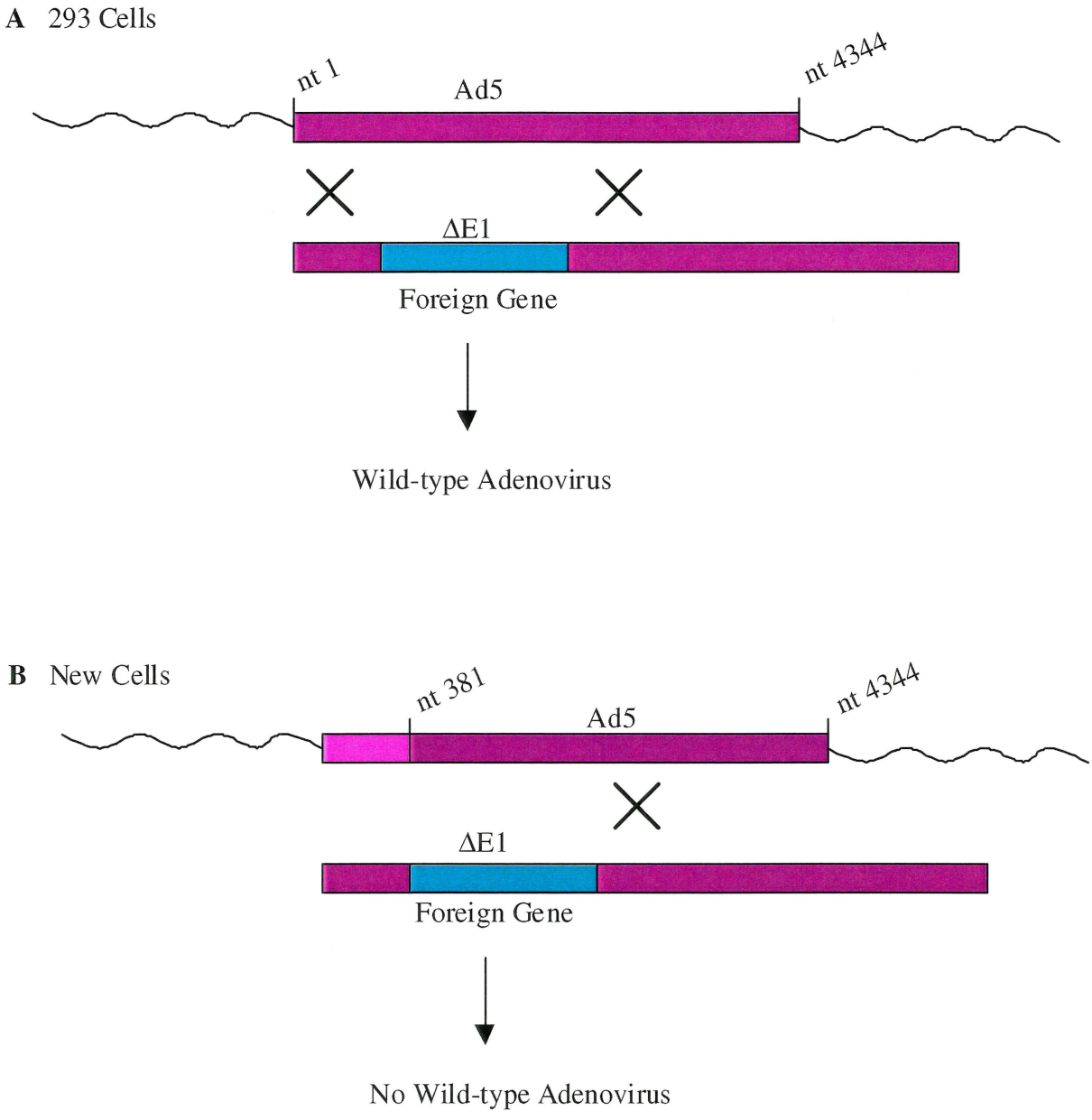


Figure 5. The interactions of packaging cell lines and recombinant ΔE1 vectors. Panel **A** shows the homologous recombination between 293 cells and adenoviral vectors to generate RCA. Panel **B** shows that the new packaging cells cannot generate a double crossover and RCA.

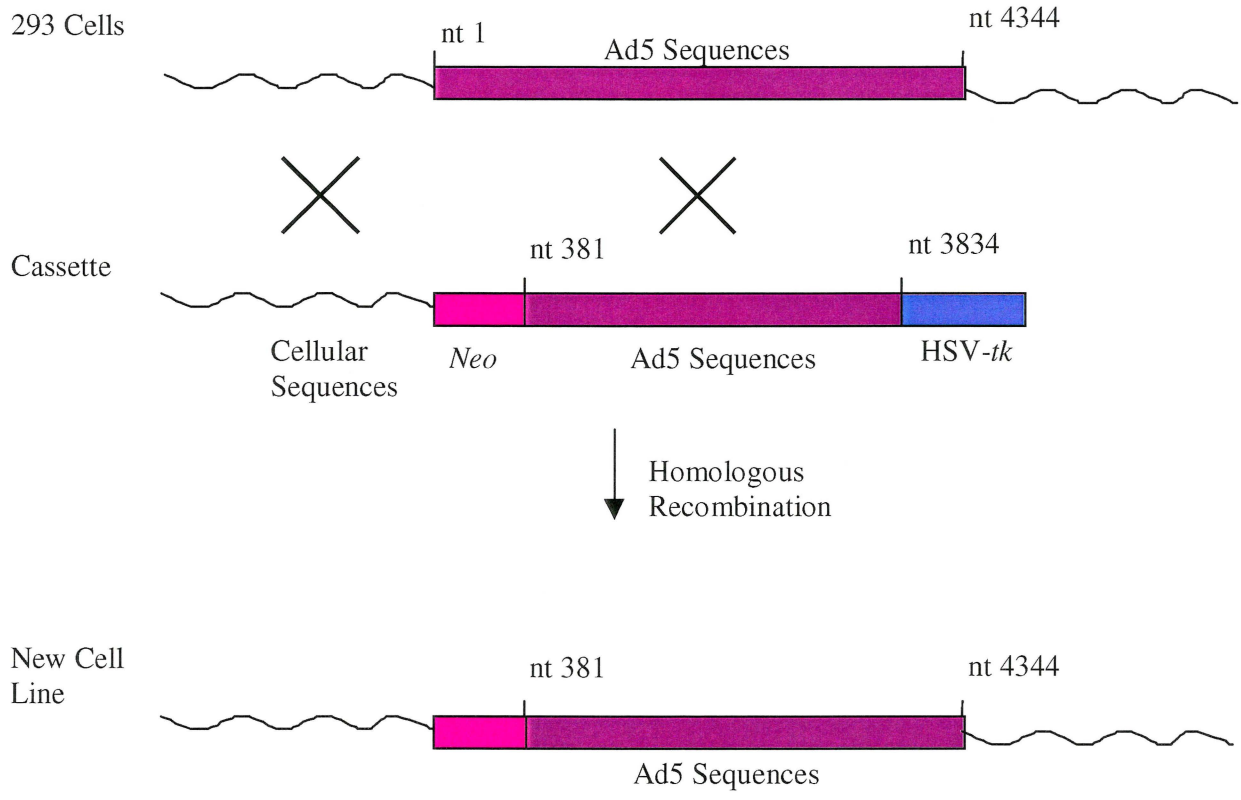


Figure 6. General outline for the design of the cassette that will be used to modify the 293 cell line (see text for details).

3.1 IDENTIFICATION AND CLONING OF THE LEFT CELLULAR-VIRAL JUNCTION IN 293 CELLS

In order to construct the cassette that will be used to modify 293 cells, it was necessary to obtain an appropriate region of homology, such as the left cellular-viral junction in the 293 cells. It has been previously established that the Ad5 sequences in 293 cells extend from nucleotides 1 to 4344 and are located in the pregnancy-specific β-1-glycoprotein 4 (PSG4) gene (Louis *et al.*, 1997). This would map the insertion of Ad5 DNA to human chromosome 19 (19q13.2). On the basis of this information primers were generated so that the appropriate region can be cloned subsequent to PCR amplification.

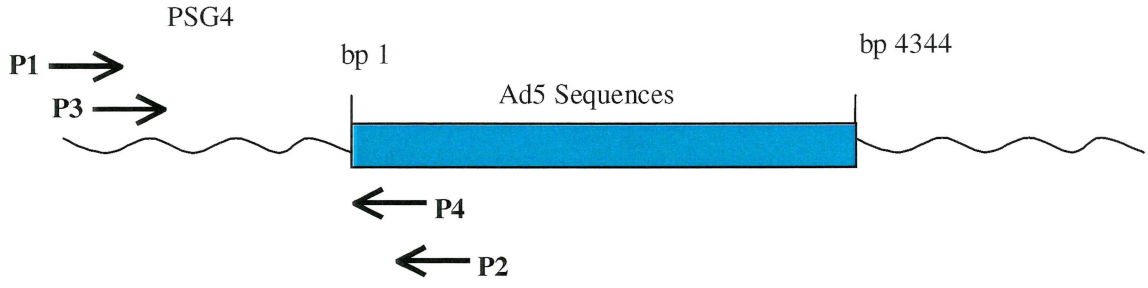
The first primer (PSG4-1) was designed to anneal from nt 1 to nt 20 of the PSG4 gene sequence of Zimmerman *et al.* (1989). This sequence can be found on Genbank (Accession # M33665) and is the sequence that Louis *et al.* (1997) used to establish that the Ad5 sequence is integrated into the PSG4 gene. The primer was designed to anneal to the extreme end of the PSG4 gene, such that the largest PCR product possible would be generated, and therefore, the largest region of homology obtained to construct the cassette. The second primer (FG293) was designed to anneal from nt 94 to nt 123 of the integrated Ad5 genome (Figure 7). The primer was generated to anneal to the adenoviral sequences in order to minimize the probability that the primer would bind randomly across the human genome. The primers were designed such that a 985 bp product would be generated.

Numerous PCRs were performed using this primer pair and genomic DNA from 293 cells as the template. In each case, the results of the PCR were similar. While the expected product was 985 bp in size, each time the PCR was performed, the product contained numerous bands resulting in a smearing pattern (Figure 8). Attempts to alter the PCR conditions to improve the results, such as lengths and temperatures of the annealing and melting steps, did not change the outcome appreciably. In response to this problem, 2 new primers were generated to attempt a

nested PCR. The third primer (PSG4Xma) was designed to anneal from nt 31 to nt 55 of the PSG4 gene sequence (Accession #M33665), and contained an *Xma*I site on the end to allow for cloning of the PCR product. This primer was designed so that it annealed at a position downstream from PSG4-1, yet still allowed for a large region of homology to be obtained in order to construct the cassette. The fourth primer (293Xba) was constructed to anneal from nt 17 to nt 40 of the Ad5 genome and contained an *Xba*I site on the end for cloning of the PCR product. In this case, the primer was designed such that it was located upstream from the primer 293FG, but again was located close enough to obtain a large amount of the cellular-viral junction for design of the cassette. The expected PCR product using the new primers was 872 bps (Figure 7).

Nested PCR was then performed using the new primer pair. First, the original PCR was performed using PSG4-1 and FG293. The resulting PCR product, which contained the numerous fragments, was then used as the template in the next PCR reaction, and the primers PSG4Xma and 293Xba were used (Figure 8). This strategy resulted in the generation of a single PCR product, which was slightly larger than the expected size of 872 bp (Figure 9). The PCR product was then digested with *Xba*I and *Xma*I for cloning into the multiple cloning site of pUC19 (Figure 10). Clones were screened with *Eco*RI and one particular clone, named p293LJ (Figure 11), was selected for further analysis by sequencing.

A Chromosome 19 (19q13.2)



B

Primer	Sequence of Primer (5' to 3')	Location of Primer*
P1: PSG4-1	GCGGGGTATATTGCAGGACC	nt 1 of PSG4
P2: FG293	ATCACACTTCCGCCACACTACTACGTCACC	nt 123 of Ad5
P3: PSG4Xma	TCCCCCCGGGGAAATACGGAACCCAGTGAGTGCCA	nt 31 of PSG4
P4: 293Xba	GCTCTAGATATTGGCTTCAATCCAAAATATGG	nt 40 of Ad5

Figure 7. Sequence and location of PCR primers used to clone the cellular-viral junction. Panel **A** shows the location of the Ad5 sequences in the human genome, as well as the location of primers 1 – 4. Panel **B** shows the sequences of primers 1 – 4 as well as their exact location in terms of the 293 genome (* position of the 5′ nucleotide).

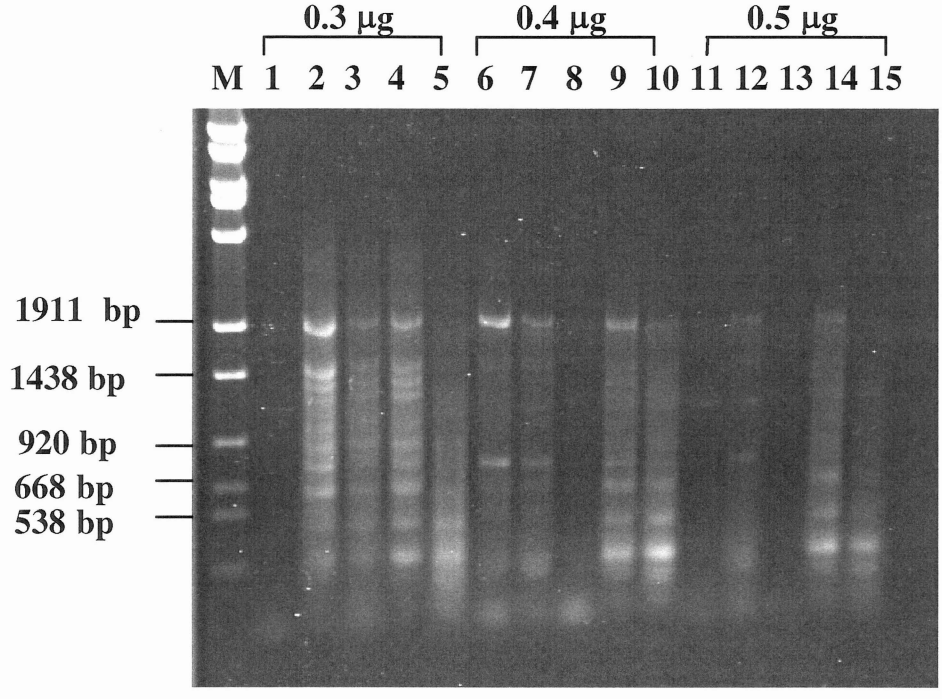


Figure 8. PCR amplification of the left cellular-viral junction in 293 cells. For this PCR the primers PSG4-1 and 293FG were used. Various PCR conditions were tested as follows: 0.3 µg of genomic DNA template (lanes 1-5); 0.4 µg of genomic DNA template (lanes 6-10); 0.5 µg of genomic DNA template (lanes 11-15); 1.0 mM $MgCl_2$ (lanes 1, 6, 11); 1.5 mM $MgCl_2$ (lanes 2, 7, 12); 2.0 mM $MgCl_2$ (lanes 3, 8, 13); 2.5 mM $MgCl_2$ (lanes 4, 9, 14); and 4.0 mM $MgCl_2$ (lanes 5, 10, 15). M denotes the Norgen N1 molecular weight marker.

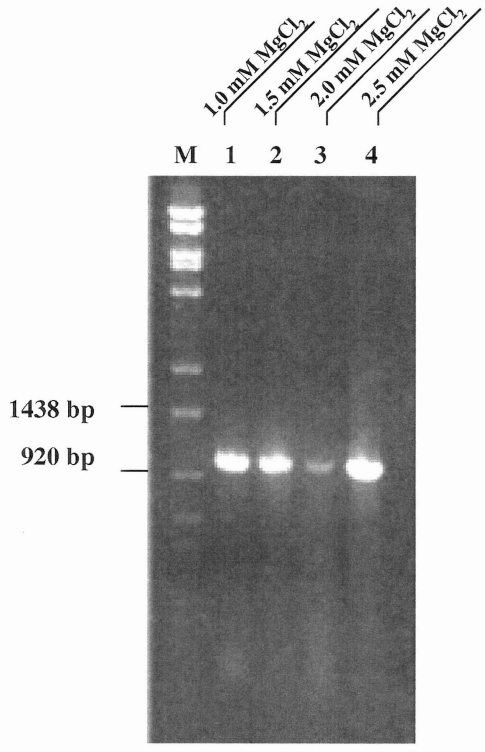


Figure 9. Nested PCR amplification of the left cellular-viral juction in 293 cells. The primers PSG4Xma and 293 Xba were used, and DNA from lane 9 in Figure 8 was used as the template. Various PCR conditions were tested as follows: 1.0 mM $MgCl_2$ (lane 1); 1.5 mM $MgCl_2$ (lane 2); 2.0 mM $MgCl_2$ (lane 3); 2.5 mM $MgCl_2$ (lane 4). M denotes the Norgen N1 molecular weight marker.

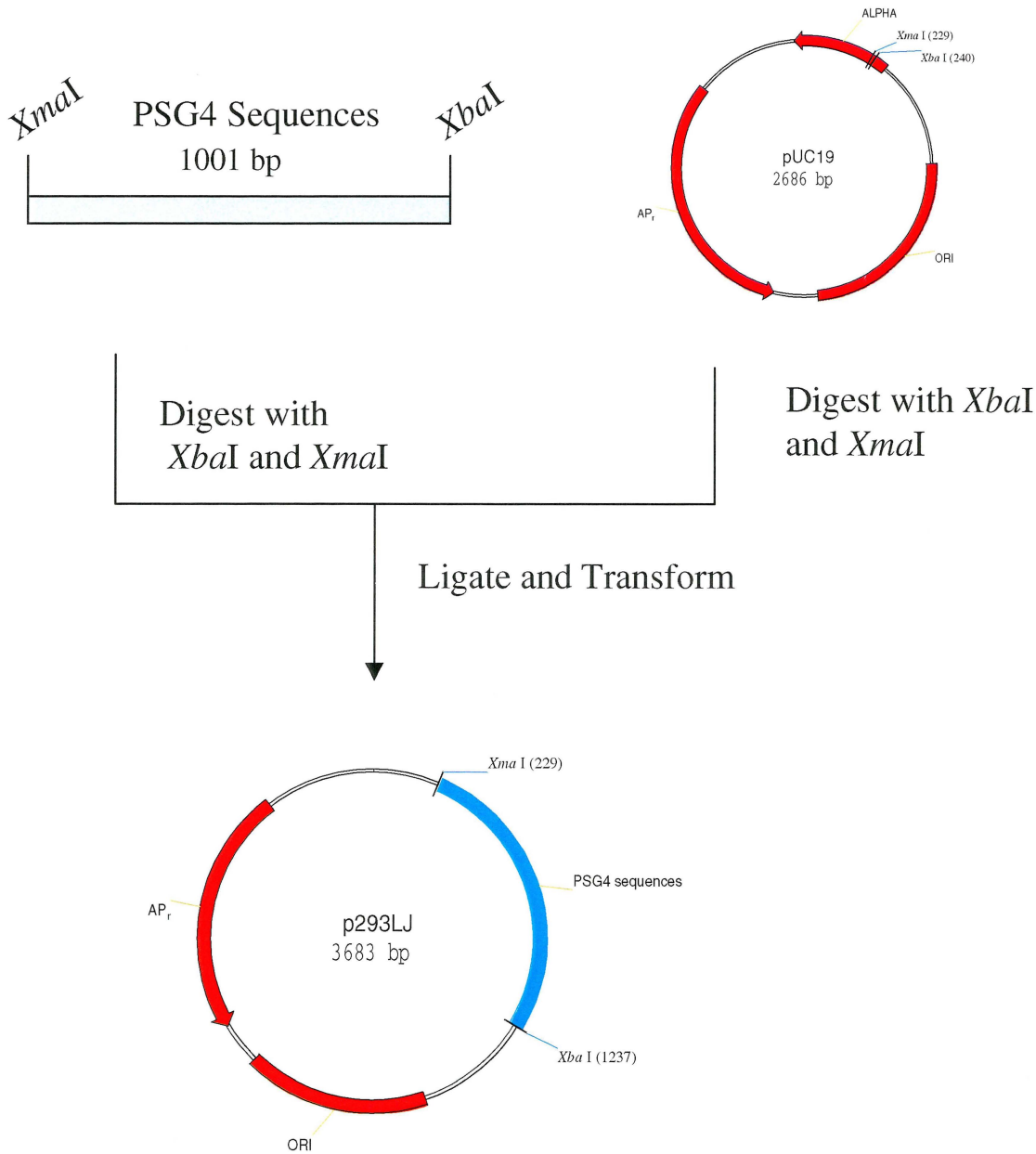
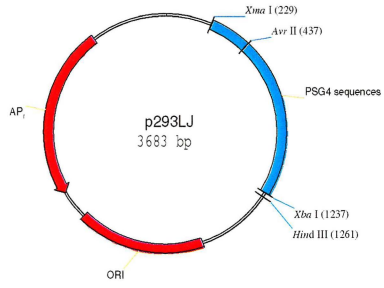


Figure 10. Diagram of the strategy to clone p293LJ (see text for details).

A



B

Restriction Enzyme	*Xma* I + *Xba* I	*Avr* II	*Hind* III
Fragment Size (bp)	2675 1008	3683	3683

C

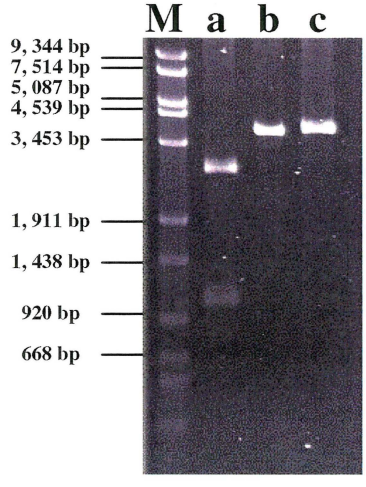


Figure 11. Confirmation of p293LJ. Panel **A** is a restriction map of p293LJ showing the relevant sites of *Xma*I, *Xba*I, *Avr*II, and *Hind*III. Panel **B** is a table showing the expected fragment sizes when the plasmid is digested with the specified enzymes. Panel **C** is a picture of the restriction fragments of p293LJ. The M denotes the Norgen N1 marker, lane a is the *Xma*I and *Xba*I digest, lane b is the *Avr*II digest and lane c is the *Hind*III digest.

3.1.1 SEQUENCE ANALYSIS OF THE LEFT CELLULAR-VIRAL JUNCTION

To precisely analyze the cloned PSG4 fragment, sequencing was carried out at Norgen Biotek Corp. (St. Catharines, ON). Alignment of sequencing results was performed using the web-based BLAST program (Altshul *et al.*, 1997) (National Center for Biotechnology Information, NCBI). As previously stated, the design of the primers to clone the junction was based on the work by Louis *et al.* (1997). Unfortunately, the sequencing results of these researchers were not published, and thus the sequence obtained in this work could not be directly compared to their sequence. However, Louis and his colleagues determined that the Ad sequences were integrated into chromosome 19q13.2, and thus, the sequence from p293LJ could be aligned with chromosome 19 (Accession # AC005392). Furthermore, a group of researchers from the University of British Columbia (UBC) (Hayashi *et al.*, 2000) have also cloned and sequenced the cellular-viral junction from 293 cells, and their results are available from the GenBank database (Accession # AF288220). Thus an alignment was performed between the three sequences; (1) chromosome 19, (2) the PSG4-Ad5 junction from UBC, and (3) the sequence obtained from p293LJ.

From the alignments, it was determined that the UBC PSG4 Ad5 sequence (AF288220) aligned with chromosome 19 for 850 base pairs to the left of the site of integration of the adenoviral sequences (Figure 12). Upon alignment of p293LJ, it was found to align with PSG4 Ad5 for 31 bp of the integrated Ad5 sequence, and then for 176 bps to the left of the site of integration (Figure 12). Furthermore, p293LJ was found to align with chromosome 19 for a stretch of 176 bps left from the site of integration, and then there was a 3928 bp gap, and the sequence then aligns with chromosome 19 for an additional 767 bps (Figure 12). If the site of integration of the adenoviral sequences is termed position 1, then this second region of alignment corresponds to chromosome 19 at positions –4103 to –4870 (Figure 12). Thus, p293LJ appears to have a deletion from position –175 to –4103, for a total of a 3928 bp deletion. The

consequences of such a deletion being present in the cell line, as well as the effect of this deletion on the strategy to generate the targeting cassette are examined in the Discussion section.

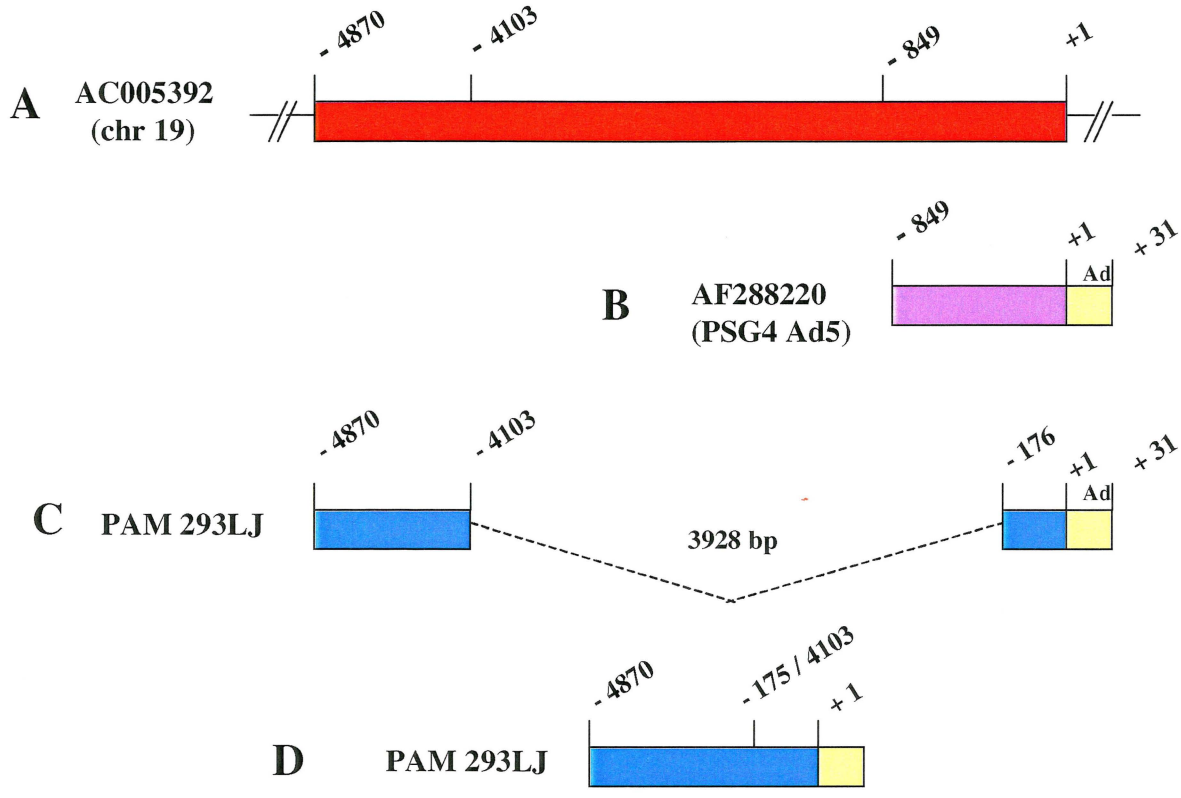


Figure 12. Diagram of the alignment of p293LJ. Panel **A** corresponds to chromosome 19 (AC005392), with the site of Ad5 integration as position +1. Panel **B** is the PSG4 Ad5 sequence (AF288220) generated by the researchers at UBC (see text). Panel **C** shows the alignment of p293LJ with both chromosome 19 and PSG4 Ad5, while panel **D** shows how p293LJ looks without alignment to the other sequences.

3.2 CONSTRUCTION OF THE CASSETTE FOR MODIFICATION OF THE 293 CELL LINE

As previously stated, a number of distinct features had to be included in the cassette and had to be taken into consideration when designing the cloning strategy for the cassette based on the double selection method of Mansour *et al.* (1988). The cassette needed to contain; (1) the cellular-viral junction as the left-end region of homology, (2) the *neo* gene to use for positive selection upon transfection into 293 cells, (3) the adenoviral genome from bp 380 to a point before bp 4344 as the right-end region of homology, and (4) the HSV-*tk* gene to use for negative selection. Due to the fact that illegitimate or non-homologous recombination is 1000X more likely to occur than homologous recombination in eukaryotic cells, the cassette was constructed in such a way as to enrich for the probability of generating homologous recombinants (Mansour *et al.*, 1988).

The overall strategy for construction of the cassette is shown in Figure 13. The details of each cloning step and the methods used to verify the intermediate plasmids are described in the following sections. Briefly, the first cloning step involved pXC38, a plasmid which contains the E1A, E1B and part of the E2 region of the Ad5 genome (Bautista *et al.*, 1991). This plasmid was modified through PCR such that the first 380 bps of the adenoviral genome were removed. Next, a linker was placed into the plasmid, and the Ad5 sequences from bp 380 to bp 4249 were subcloned into pUC19. Subsequently, the *neo* gene from pSV2neo (Southern and Berg, 1982) was cloned into the plasmid p293LJ, and the *neo* gene and the cellular sequences were cloned together as a unit into the pUC-based plasmid containing the adenovirus sequences. Lastly, the HSV-*tk* gene from pTK173 was subcloned into pUC19, and finally, the *tk* gene was cloned from this plasmid into the adenovirus containing plasmid to generate the modifying cassette PAM2.

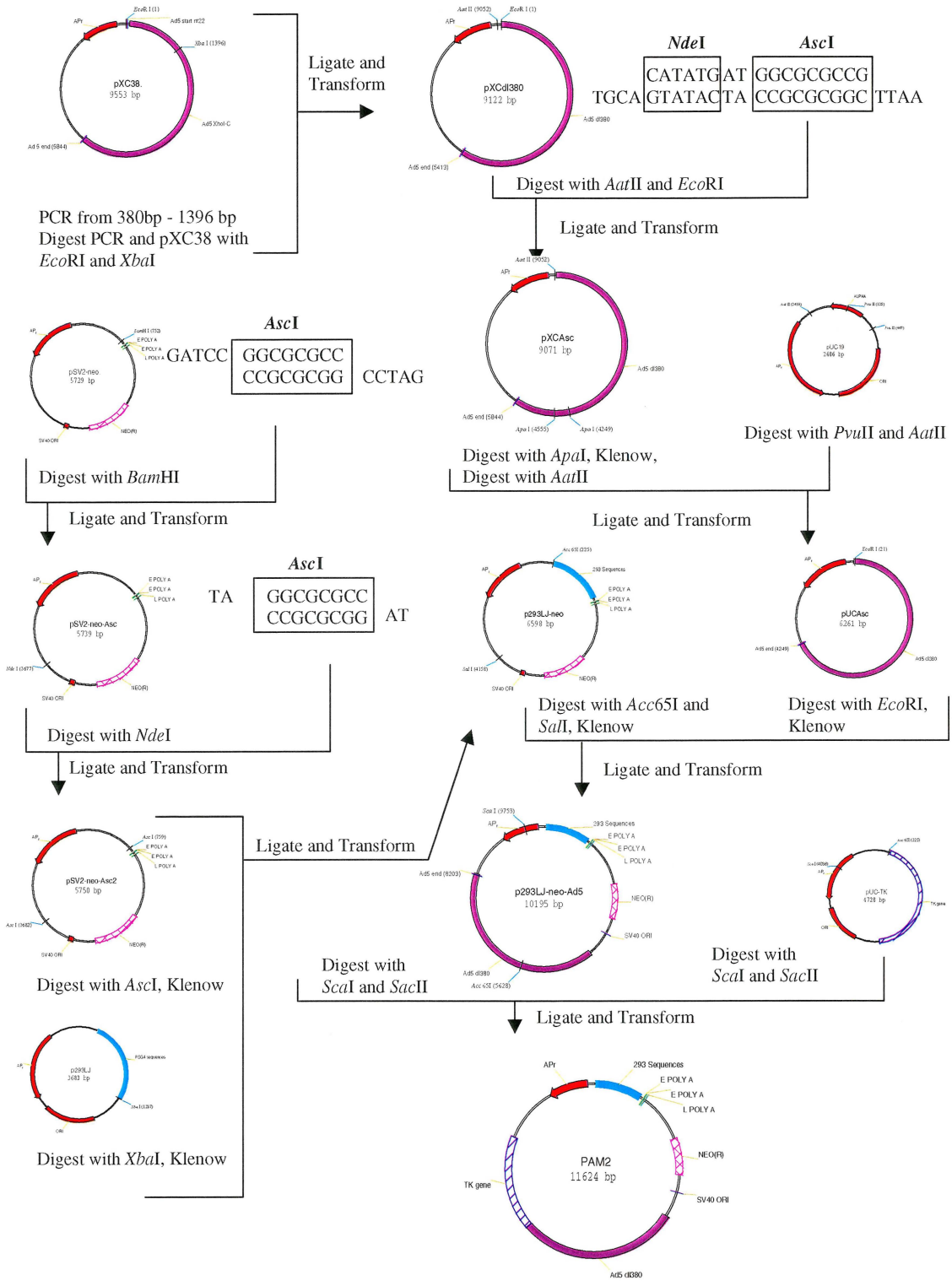


Figure 13. Overall strategy for the construction of the modification cassette PAM2 (see subsequent sections for clarification of each step).

3.2.1 CONSTRUCTION OF pXCdl380

The first step in the construction of the cassette involved generating the specific 380-bp deletion. There were a number of reasons for excluding the first 380 bps of the adenovirus genome. As previously stated, it has been determined that replication competent adenoviruses (RCA) are generated through recombination between E1 replacement vectors and homologous sequences in the 293 cell line (Hehir *et al.*, 1996). The mechanism for generating replication competent revertants in which E1 is reconstituted requires a minimum of two crossovers, one occurring on either side of the deleted E1. Thus, by removing the adenovirus sequences in the cell line that are homologous to the adenovirus sequences located on the left-hand side of the transgene, a double crossing-over event may be prevented from occurring. Elimination of one crossover (in this case, on the left side of E1) essentially prevents recovery of revertants as predicted by recombination models (Hehir *et al.*, 1996).

Second, the adenovirus genome is well characterized in this region of interest (Figure 14). It is known that an inverted terminal repeat (ITR) region is located in the first 100 base pairs of the genome (reviewed in Horitz, 1990). This 380 bp region also contains elements involved in Ad5 E1A transcriptional control, including enhancer elements (Hearing and Shenk, 1983) and *cis*-acting packaging elements (Hearing *et al.*, 1987), both located between bp 194 and 358. The core elements of the promoter, the CAAT box and the TATA box are located outside of this region, with the TATA beginning at bp 469 (Osbourne *et al.*, 1982; Hearing and Shenk, 1983). The E1A cap site is determined by the TATA box and is located at position 499 of the Ad5 genome. Deletion of the first 380 base pairs of Ad5 would result in the removal of the ITR along with the enhancer and packaging sequences, without removing the promoter elements themselves. Thus it was not necessary to replace the E1 promoter with a different promoter, which would have resulted in the introduction of additional sequences. These additional sequences might then serve as potential sites for homologous recombination between adenoviral

vectors and the cell line, and thus may have been additional locations of recombination between the cell line and the vectors.

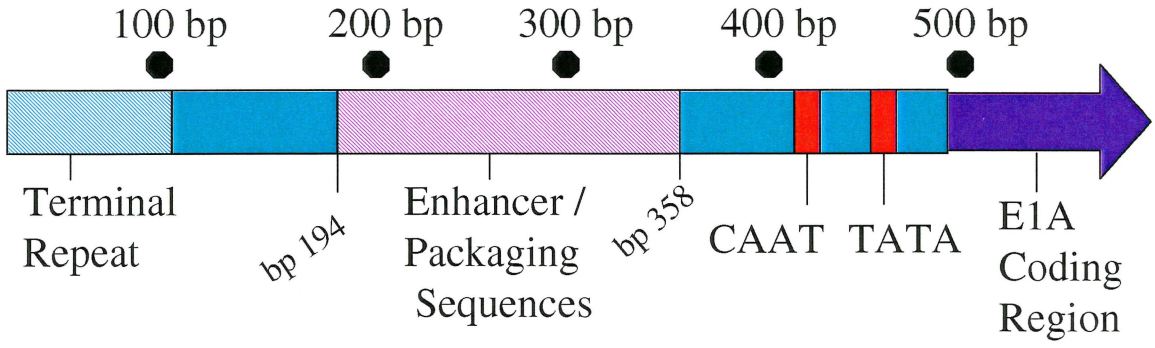


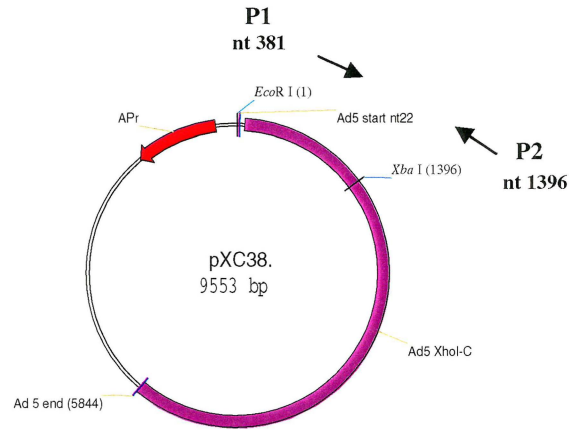
Figure 14: Diagram of the 5′ region of the Ad5 genome.

In order to generate the 380 bp deletion, PCR was performed on the plasmid pXC38. This plasmid is 9553 bp in size and contains the adenovirus genome from nucleotide 1 to 5866. The first PCR primer generated was Ad5 381 Eco, and this primer annealed from nt 437 to 454 of pXC38, or nt 381 to 398 of the Ad5 genome. The primer was generated such that it had an *Eco*RI site tagged to the end, which could be used to clone the PCR product. The second PCR primer generated was Ad5 1346, which annealed from bp 1378 to 1402 of the plasmid pXC38, or nt 1322 to 1346 of the Ad genome. This primer was generated to contain an *Xba*I site on the end, again to be used in cloning the PCR product (Figure 15).

PCR was performed using the Expand™ High Fidelity PCR System (Boehringer Mannheim), with pXC38 as the template and the primers Ad5 381 Eco and Ad5 1346. The Expand™ High Fidelity PCR System was used because it has 3′-5′ proofreading activity, resulting in a 3-fold increased fidelity of DNA synthesis when compared to that of Taq DNA

polymerase, as claimed by the manufacturer. Therefore, the introduction of errors that may interfere with the expression of the E1 region in the cell line would be reduced. The resulting PCR product was 965 bp in size (Figure 15). The PCR product was digested with *Eco*RI and *Xba*I and was cloned back into pXC38, which was also digested with the same restriction enzymes. This cloning step resulted in the generation of the plasmid pXCdl380, which contained the Ad5 genome from base pair 380 to base pair 5866 (Figure 16). The restriction enzyme confirmation of the plasmid is shown in Figure 17.

A



B

Primer	Sequence of Primer (5' to 3')	Location of Primer*
P1: Ad5 381 Eco	CCGGAATTCAGACTCGCCCAGGTGTTT	nt 437 of pXC38
P2: Ad5 1346	TCTCTAGACACAGGTGATGTCGGGC	nt 1402 of pXC38

C

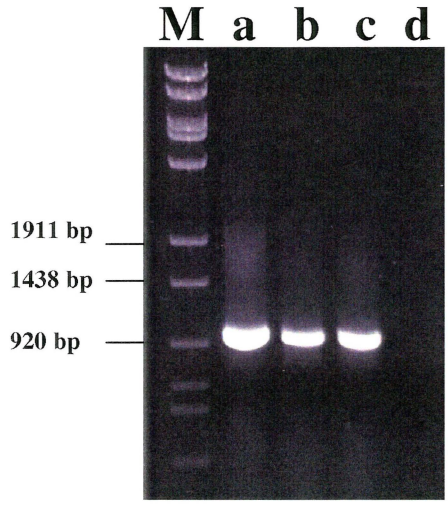


Figure 15. Amplification of the Ad5 genome from nt 381 – nt 1396. Panel **A** shows the approximate location of the primers on the plasmid pXC38. Panel **B** shows the sequence of the primers, and their exact locations on pXC38 (* position of the 5′ nucleotide). Panel **C** shows the agarose gel of the PCR products. Various PCR conditions were tested as follows: 0.1 µg of pXC38 (lane a); 0.3µg of pXC38 (lane b); 0.5 µg of pXC38 (lane c). Lane d is a negative control using pUC19, and M denotes the N1 Norgen molecular weight marker

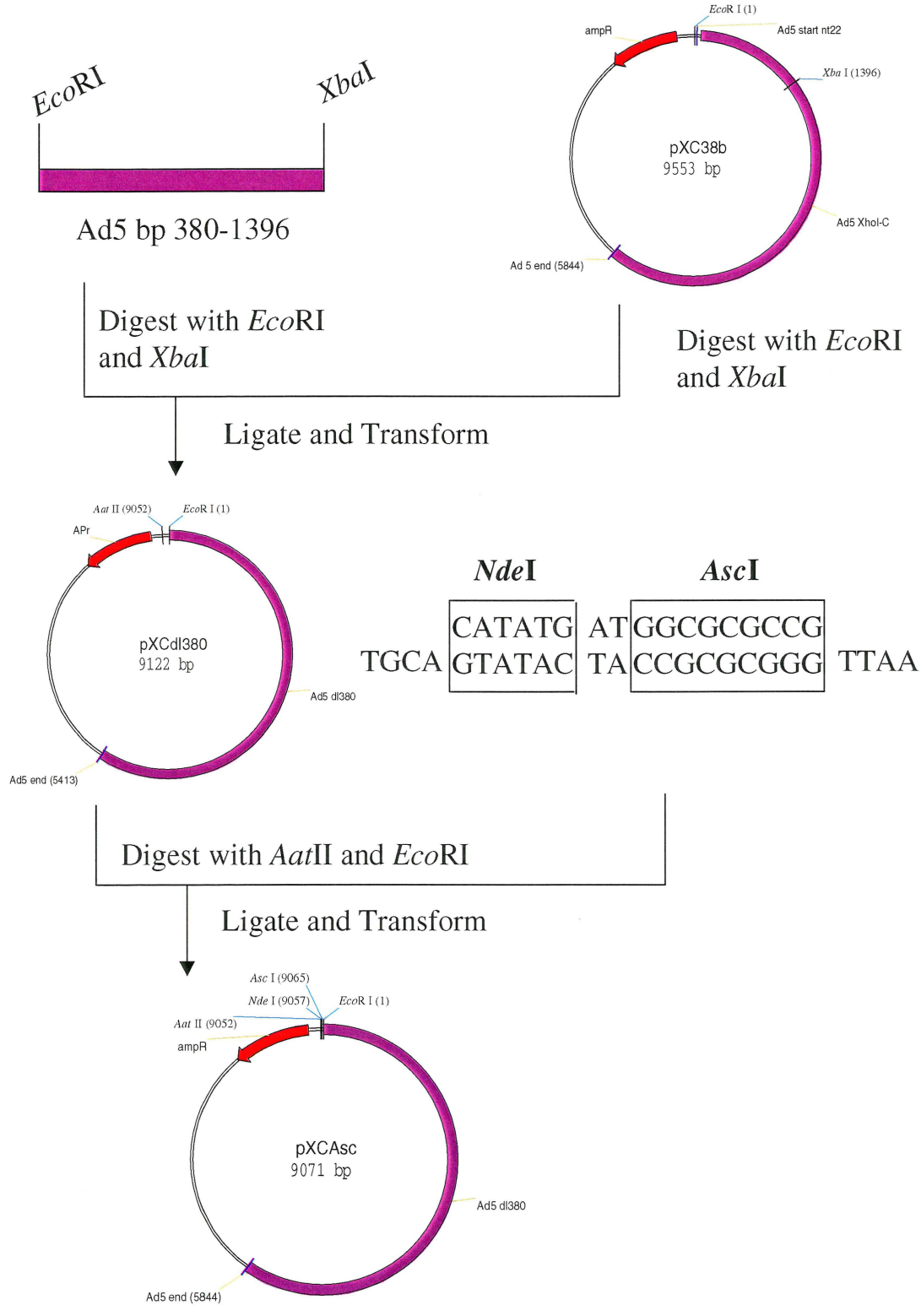
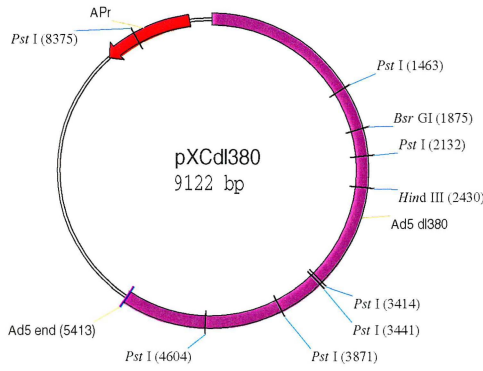


Figure 16. Diagram of the strategy to clone pXCdl380 and pXCAsc (see text for details).

A



B

Restriction Enzyme	*Bsr* GI	*Pst* I	*Hind* III
Fragment Size (bp)	9122	3771 2210 1282 733 669 430 27	9122

C

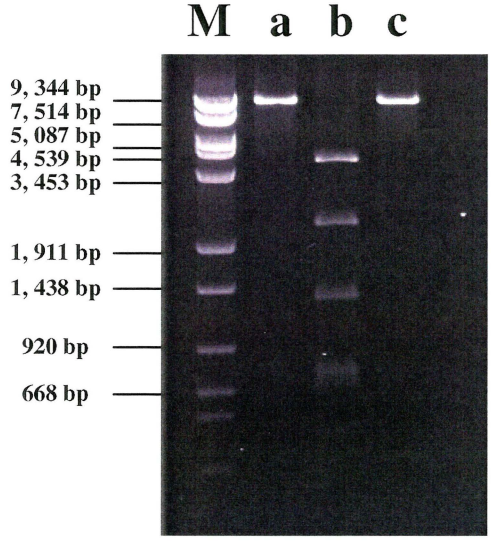
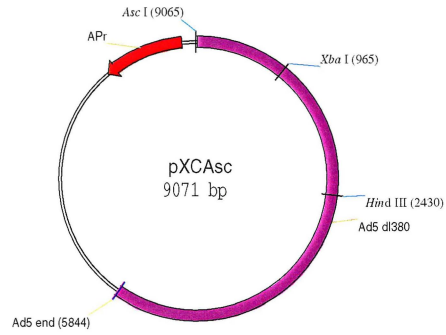


Figure 17. Confirmation of pXCdl380. Panel **A** is a restriction map of pXCdl380 showing the relevant sites of *Bsr*GI, *Pst*I, and *Hind*III. Panel **B** is a table showing the expected fragment sizes when the plasmid is digested with the specified enzymes. Panel **C** is a picture of the restriction fragments of pXCdl380. The M denotes the Norgen N1 marker, lane a is the *Brs*GI digest, lane b is the *Pst*I digest and lane c is the *Hind*III digest.

3.2.2 CONSTRUCTION OF pXCAsc

The second step in the construction of the cassette PAM2 involved cloning a linker containing unique sites into the plasmid pXCdl380, directly in front of the adenoviral sequences. The linker was designed such that it had an *Eco*RI tail on one end and an *Aat*II tail on the other end, with internal *Nde*I and *Asc*I sites (Figure 16). The strategy to clone the linker involved digesting pXCdl380 with *Eco*RI and *Aat*II, inactivating the enzymes with heat, and then ligating the digested plamid to the prepared linker (Figure 16). This procedure resulted in the generation of the plasmid pXCAsc, which contained the unique *Nde*I and *Asc*I sites that could then be used for cloning additional fragments required in the cassette. The confirmation of the plasmid's structure is shown in Figure 18.

A



B

Restriction Enzyme	*Asc* I	*Asc* I + *Bgl* II	*Asc* I + *Hind* III
Fragment Size (bp)	9071	6111 2960	6635 2436

C

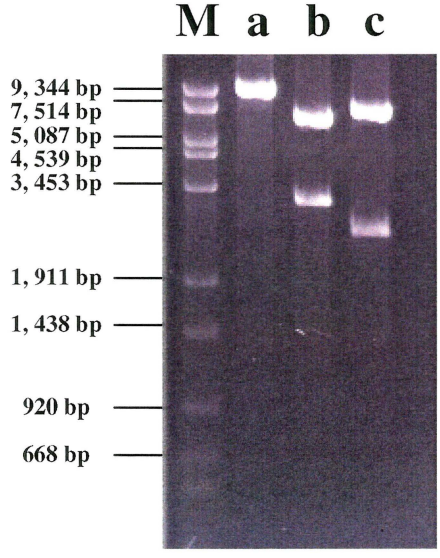


Figure 18. Confirmation of pXCAsc. Panel **A** is a restriction map of pXCAsc showing the relevant sites of *Asc*I, *Bgl*II, and *Hind*III. Panel **B** is a table showing the expected fragment sizes when the plasmid is digested with the specified enzymes. Panel **C** is a picture of the restriction fragments of pXCAsc. The M denotes the Norgen N1 marker, lane a is the *Asc*I digest, lane b is the *Asc*I and *Bgl*II digest and lane c is the *Asc*I and *Hind*III digest.

3.2.3 CONSTRUCTION OF pUCAsc

The next step in the construction of the cassette involved cloning the pXCAsc fragment containing the Ad5 genome with the desired deletion into pUC19. The reasons for this step were two-fold. First of all, by cloning pXCAsc into pUC19, the copy number would be increased, and therefore it would be easier to obtain larger amounts of the final construct in order to perform the transfections. Secondly, the linker was designed such that it contained unique restriction enzyme sites which would then be convenient for cloning additional pieces of the cassette. However, it was determined after cloning in the linker that an *Nde*I site was actually present in the backbone of the plasmid, and thus the site that was contained within the linker was not unique. Therefore by performing the subcloning step, the *Nde*I site in the linker would become unique.

The strategy for the cloning of pUCAsc is shown in Figure 19. The subcloning was accomplished by first digesting pXCAsc with the restriction enzyme *Apa*I, and then heat inactivating the enzyme. The ends were then filled in with Klenow polymerase, such that blunt ends were generated. The linearized plasmid was then digested with *Aat*II, and again the enzyme was heat inactivated. Simultaneously, the plasmid pUC19 was double digested with *Aat*II and *Pvu*II. The resulting fragments were run on an agarose gel, and the 1,991 bp pUC19 sub-fragment was purified from the gel. The pUC19 fragment was then ligated to the pXCAsc fragments, and the resulting plasmids were screened. Restriction enzyme analysis verified that the plasmid pUCAsc was generated (Figure 20). This plasmid could then be used in subsequent cloning steps in order to generate the final construct for modification of the 293 cells.

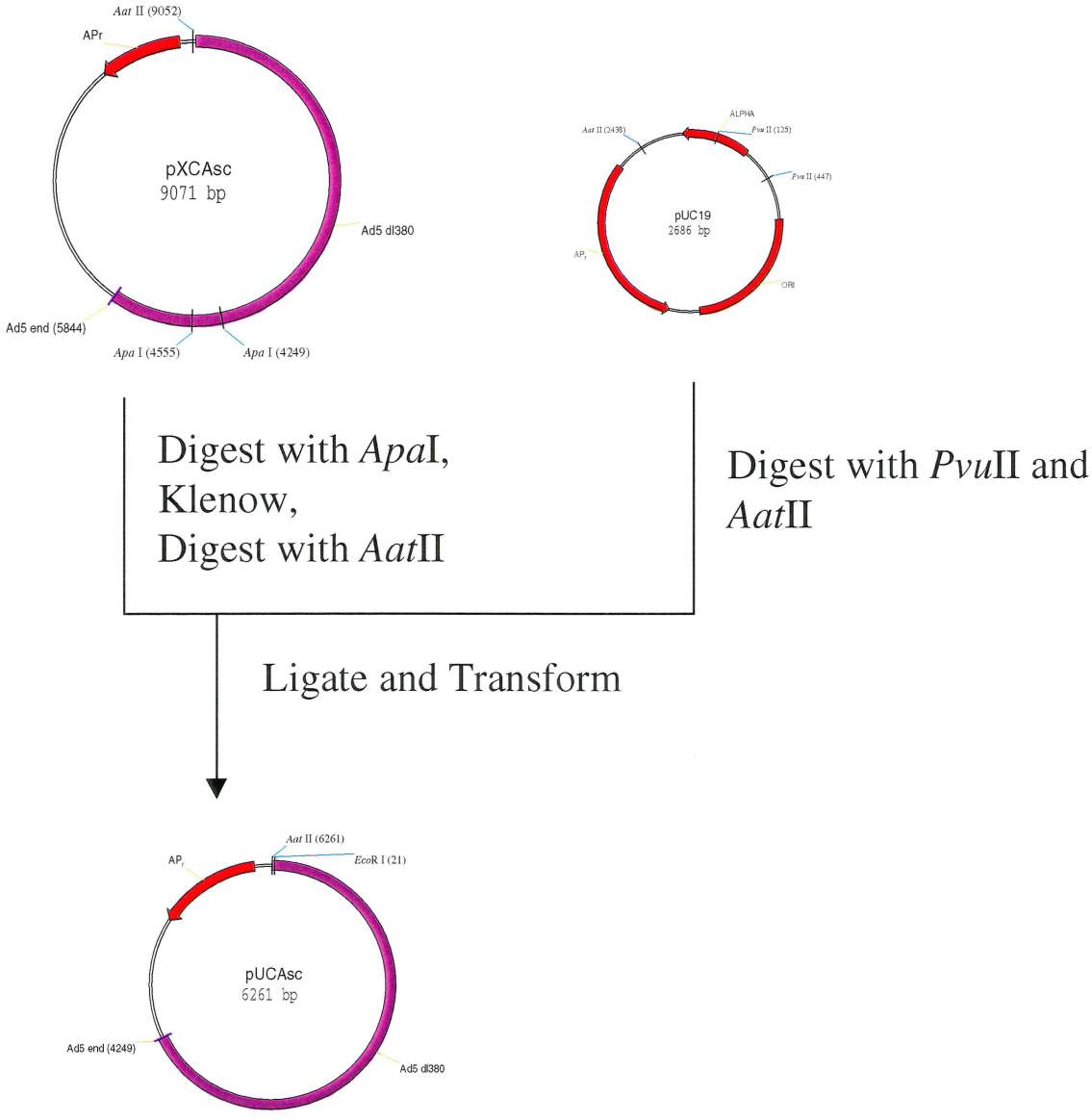
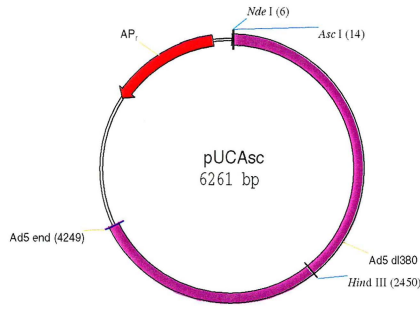


Figure 19. Diagram of the strategy to clone pUCAsc (see text for details).

A



B

Restriction Enzyme	*Nde* I	*Asc* I	*Asc* I + *Hind* III
Fragment Size (bp)	6261	6261	3825 2436

C

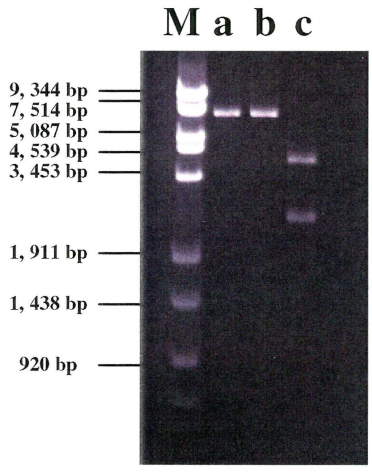


Figure 20. Confirmation of pUCAsc. Panel **A** is a restriction map of pUCAsc showing the relevant sites of *Nde*I, *Asc*I, and *Hind*III. Panel **B** is a table showing the expected fragment sizes when the plasmid is digested with the specified enzymes. Panel **C** is a picture of the restriction fragments of pUCAsc. The M denotes the Norgen N1 marker, lane a is the *Nde*I digest, lane b is the *Asc*I digest and lane c is the *Asc*I and *Hind*III digest.

3.2.4 CONSTRUCTION OF pSV2-neo-Asc and pSV2-neo-Asc2

The next cloning steps involved the plasmid pSV2neo, which contains the *neo* gene from Tn5 under control of the SV40 early promoter, as well as a polyadenylation signal for expression in mammalian cells (Southern and Berg, 1982). The *neo* gene confers resistance to the antibiotic kanamycin in bacteria, and to the amino-glycoside antibiotic G418 in mammalian cells. For ease of cloning, *Asc*I linkers were inserted on either side of the *neo* gene and its accompanying regulatory and expression sequences.

In order to construct pSV2-neo-Asc, a linker with *Bam*HI sites on both ends and an internal *Asc*I site was designed and synthesized. Subsequently, pSV2-neo was digested with *Bam*HI, the enzyme was removed, and the linker was ligated to the linearized plasmid (Figure 21). This effectively resulted in converting the *Bam*HI site to an *Asc*I site. The structure of the desired plasmid is verified in Figure 22.

The construction of pSV2-neo-Asc2 involved similar steps. However in this case, the *Asc*I linker was designed with *Nde*I sites on both ends. The plasmid pSV2-neo-Asc was then digested with *Nde*I, and the *Asc*I linker was ligated to the digested plasmid (Figure 21). The plasmid pSV2-neo-Asc2 was verified through restriction enzyme analysis (Figure 23). The resulting plasmid was similar to pSV2-neo, with the exception that the *neo* gene was now flanked by *Asc*I sites. Thus, it was a simple one-step procedure to clone out the *neo* gene, by digesting pSV2-neo-Asc2 with *Asc*I.

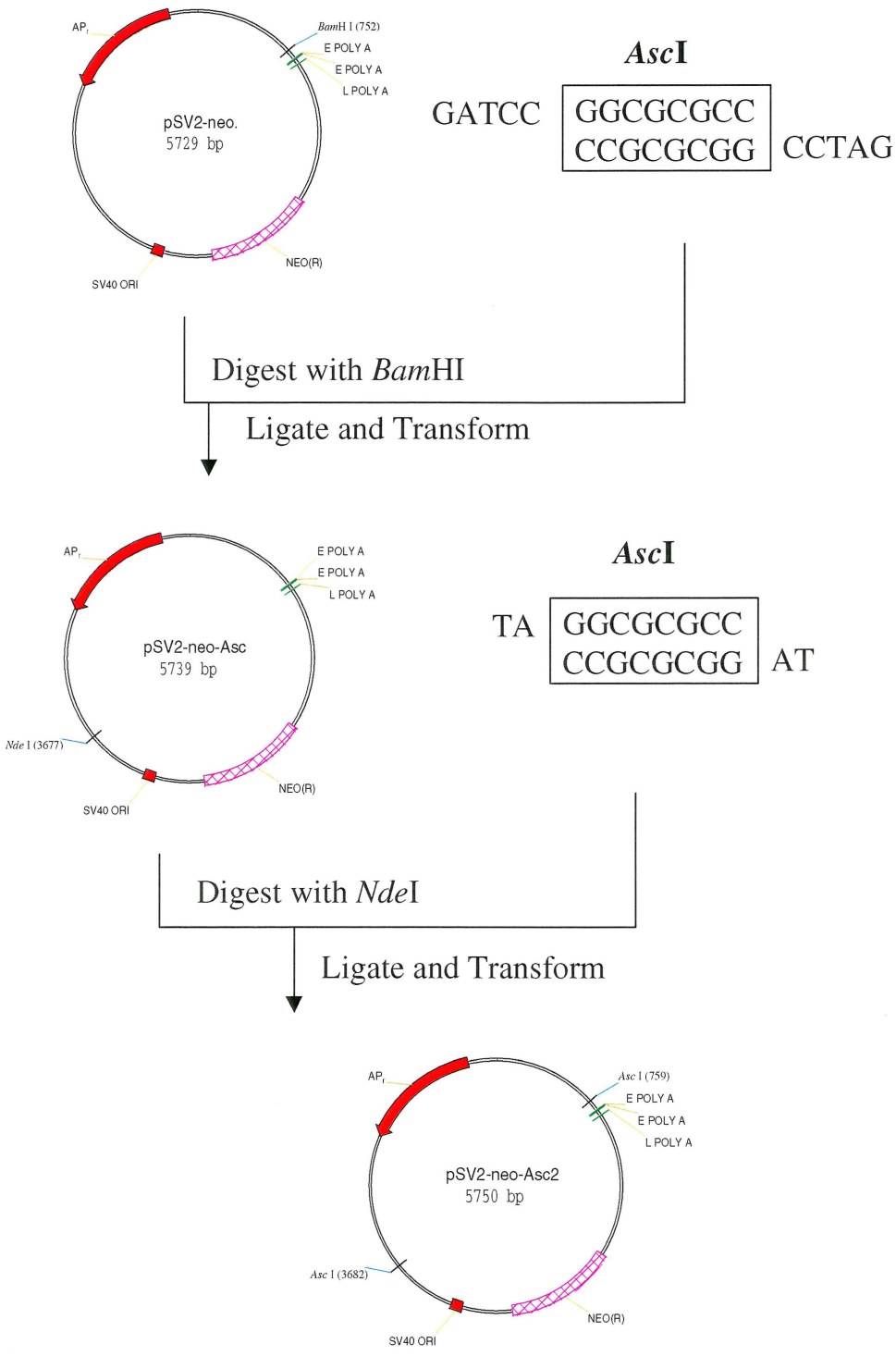
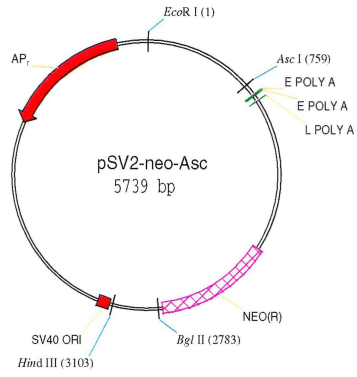


Figure 21. Diagram of the strategy to construct pSV2-neo-Asc and pSV2-neo-Asc2 (see text for details).

A



B

Restriction Enzyme	*Asc* I + *Hind* III	*Asc* I + *Eco* RI	*Asc* I + *Bgl* II
Fragment Size (bp)	3395 2344	4981 758	3715 2024

C

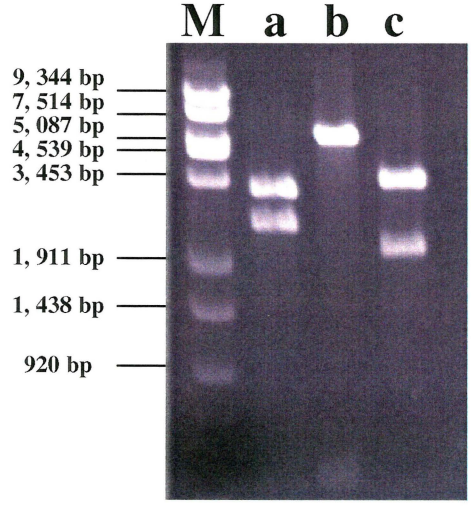
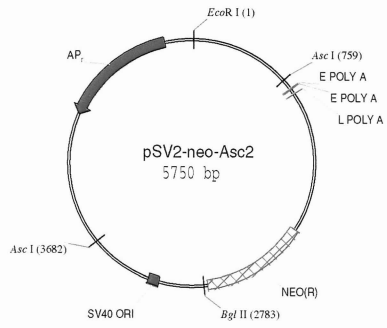


Figure 22. Confirmation of pSV2-neo-Asc. Panel **A** is a restriction map of pSV2-neo-Asc showing the relevant sites of *Asc*I, *Hind*III, *Eco*RI and *Bgl*II. Panel **B** is a table showing the expected fragment sizes when the plasmid is digested with the specified enzymes. Panel **C** is a picture of the restriction fragments of pSV2-neo-Asc. The M denotes the Norgen N1 marker, lane a is the *Asc*I and *Hind*III digest, lane b is the *Asc*I and *Eco*RI digest and lane c is the *Asc*I and *Bgl*II digest.

A



B

Restriction Enzyme	*Asc* I	*Asc* I + *Eco* RI	*Asc* I + *Bgl* II
Fragment Size (bp)	2923 2827	2923 2069 758	2827 2024 899

C

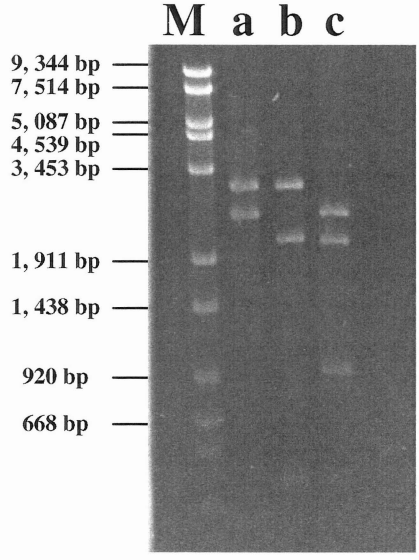


Figure 23. Confirmation of pSV2-neo-Asc2. Panel **A** is a restriction map of pSV2-neo-Asc2 showing the relevant sites of *Asc*I, *Eco*RI and *Bgl*II. Panel **B** is a table showing the expected fragment sizes when the plasmid is digested with the specified enzymes. Panel **C** is a picture of the restriction fragments of pSV2-neo-Asc2. The M denotes the Norgen N1 marker, lane a is the *Asc*I digest, lane b is the *Asc*I and *Eco*RI digest and lane c is the *Asc*I and *Bgl*II digest.

3.2.5 CONSTRUCTION OF p293LJ-neo

The next cloning step in the construction of the gene targeting cassette involved the plasmid pSV2-neo-Asc2, as well as the plasmid p293LJ. As stated previously, p293LJ contains the cellular-viral sequences from the 293 cells cloned into pUC19. This cloning step involved removing the *neo* gene from pSV2-neo-Asc2 and inserting it next to the cellular sequences (Figure 24). In order to accomplish this, p293LJ was digested with *Xba*I and then treated with Klenow polymerase in order to generate blunt ends. Next, the plasmid pSV2-neo-Asc2 was digested with *Asc*I, followed by treatment with Klenow polymerase, again generating blunt ends. The fragment containing the *neo* gene was then blunt-end cloned into the plasmid p293LJ. In order to facilitate selection, the transformed bacteria containing the plasmids for screening were plated on agar plates containing both ampicillan and kanamycin. The resulting plasmid, p293LJ-neo, contained the cellular-viral junction sequences followed by the *neo* gene, complete with all regulatory sequences necessary for expression in mammalian cells. This plasmid was confirmed by digestion with several indicative restriction enzymes, which not only confirmed the integrity of the plasmid but also allowed for the determination of the orientation of the *neo* gene (Figure 25).

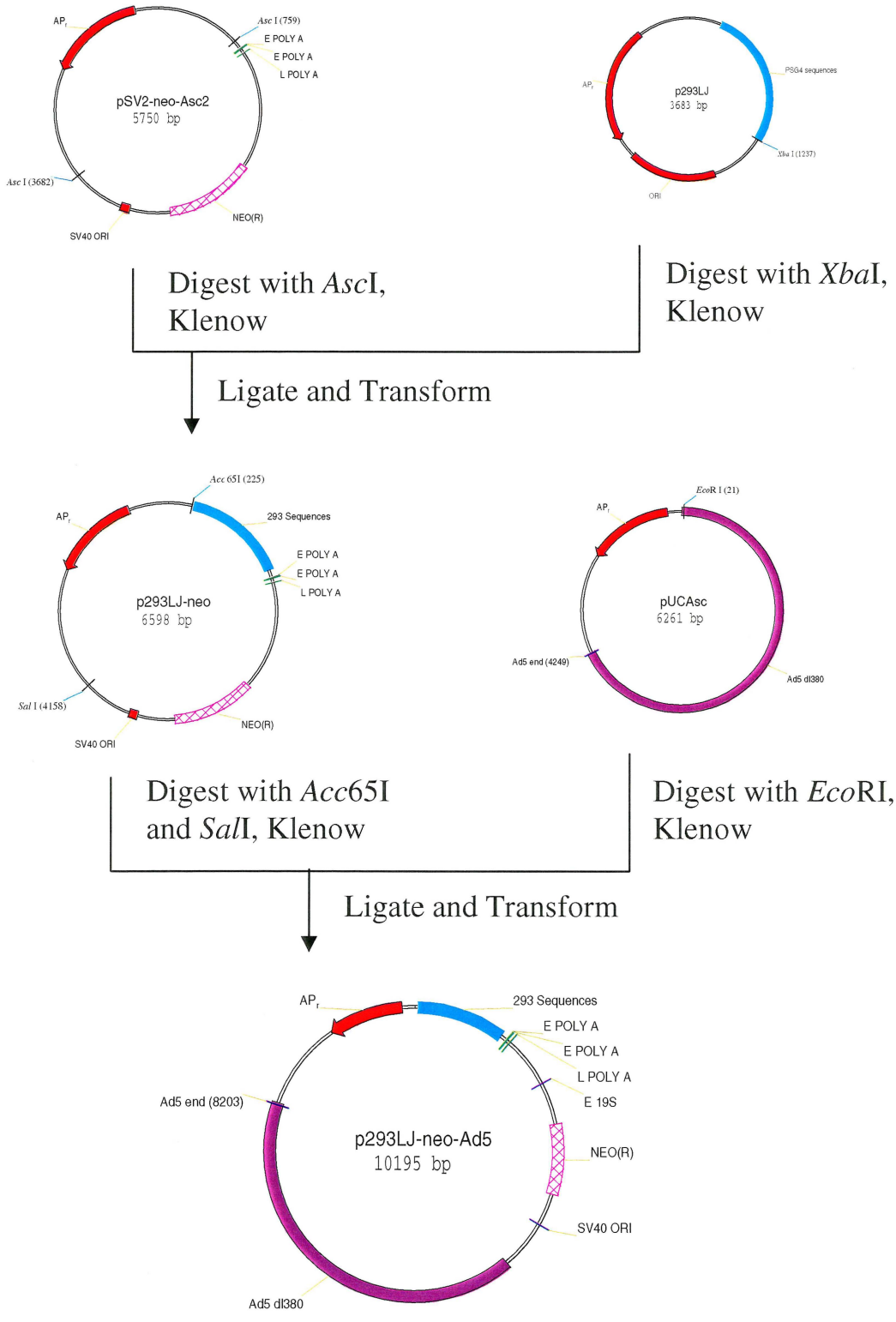
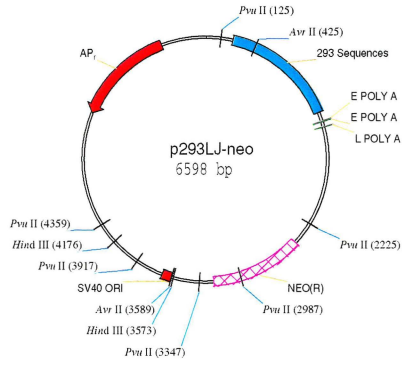


Figure 24. Diagram of the strategy to clone p293LJ-neo and p293LJ-neo-Ad5 (see text for details).

A



B

Restriction Enzyme	*Hind* III	*Pvu* II	*Avr* II
Fragment Size (bp)	5995 603	2364 2100 762 570 442 360	3434 3164

C

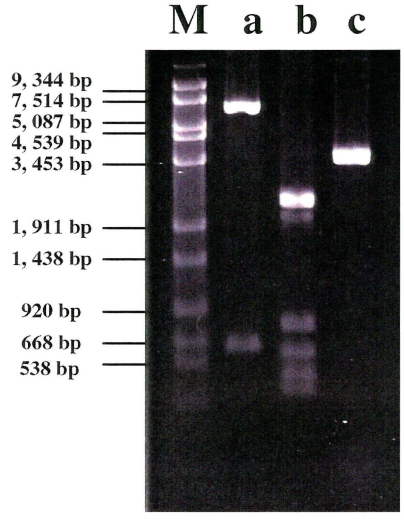
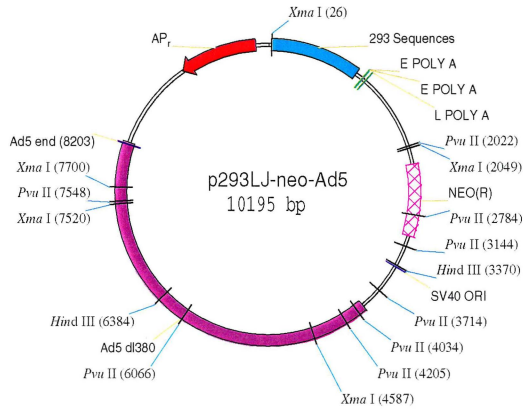


Figure 25. Confirmation of p293LJ-neo. Panel **A** is a restriction map of p293LJ-neo showing the relevant sites of *Hind*III, *Pvu*II and *Avr*II. Panel **B** is a table showing the expected fragment sizes when the plasmid is digested with the specified enzymes. Panel **C** is a picture of the restriction fragments of p293LJ-neo. The M denotes the Norgen N1 marker, lane a is the *Hind*III digest, lane b is the *Pvu*II digest, and lane c is the *Avr*II digest.

3.2.6 CONSTRUCTION OF p293LJ-neo-Ad5

The previously constructed plasmid pUCAsc was then used along with the plasmid p293LJ-neo to generate the next intermediate clone. This step involved taking the cellular-viral sequences together with the *neo* gene, and cloning these elements into pUCAsc directly before the adenoviral sequences (Figure 24). This strategy was accomplished by first digesting pUCAsc with *Eco*RI, and then treating the linearized plasmid with Klenow polymerase. Next, the plasmid p293LJ-neo was digested with *Acc*65I and *Sal*I and was then also treated with Klenow to generate blunt ends. The resultant two fragments were then run on an agarose gel, and the larger fragment containing the cellular sequences and the *neo* gene was purified from the gel. This blunt-end fragment was then ligated into the blunt-end plasmid pUCAsc, resulting in the generation of p293LJ-neo-Ad5. The plasmid was verified through restriction enzyme analysis (Figure 26). The resulting plasmid contained the cellular-viral junction, the *neo* gene, and the adenoviral sequences from bp 380 to bp 4249.

A



B

Restriction Enzyme	*Hind* III	*Xma* I	*Pvu* II
Fragment Size (bp)	7181	2933	4669
	3014	2538	1861
		2521	1482
		2023	762
		180	570
			360
			320
			171

C

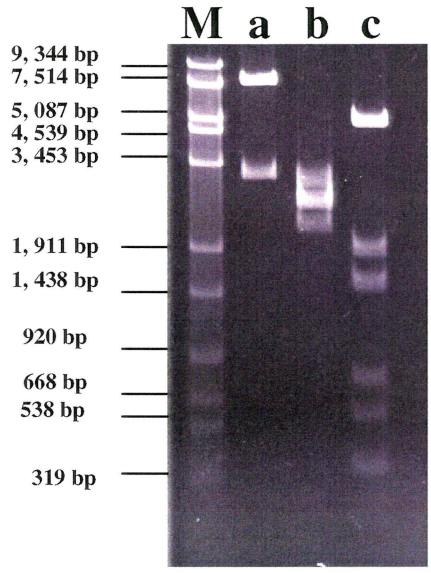


Figure 26. Confirmation of p293LJ-neo-Ad5. Panel **A** is a restriction map of p293LJ-neo-Ad5 showing the relevant sites of *Hind*III, *Xma*I and *Pvu*II. Panel **B** is a table showing the expected fragment sizes when the plasmid is digested with the specified enzymes. Panel **C** is a picture of the restriction fragments of p293LJ-neo-Ad5. The M denotes the Norgen N1 marker, lane a is the *Hind*III digest, lane b is the *Xma*I digest, and lane c is the *Pvu*II digest.

3.2.7 CONSTRCUTION OF pUC-TK

The next piece of the cassette that needed to be cloned was the *tk* gene, which was to be used for negative selection. The source of the gene was the plasmid pTK173, which contains the entire HSV-*tk* gene (*Pvu*II) fragment cloned into pBR322 at the *Pvu*II site (reviewed in Haj-Ahmad and Graham, 1986). This *Pvu*II fragment of the HSV-*tk* gene contains all the promoter and enhancer elements necessary for *tk* gene expression in mammalian cells (Smiley, 1985; Jones *et al.*, 1985). To facilitate the cloning of the *tk* gene into the plasmid p293LJ-neo-Ad5, the gene was first sub-cloned into pUC19 (Figure 27). First the plasmid pTK173 was digested with *Pvu*II, and then treated with shrimp alkaline phosphatase. Next, pUC19 was digested with *Hinc*II, and then the *Pvu*II fragment from pTK173 was cloned into the plasmid. Screening for the desired plasmid was facilitated by the use of X-gal containing plates and the process of α-complementation. The resulting plasmid, pUC-TK, contained the *tk* gene within the *Hinc*II site of pUC19. This plasmid was verified through digestion with a number of indicative restrcition enzymes, which not only confirmed the structure of the plasmid but also the orientation of the *tk* gene within the plasmid (Figure 28).

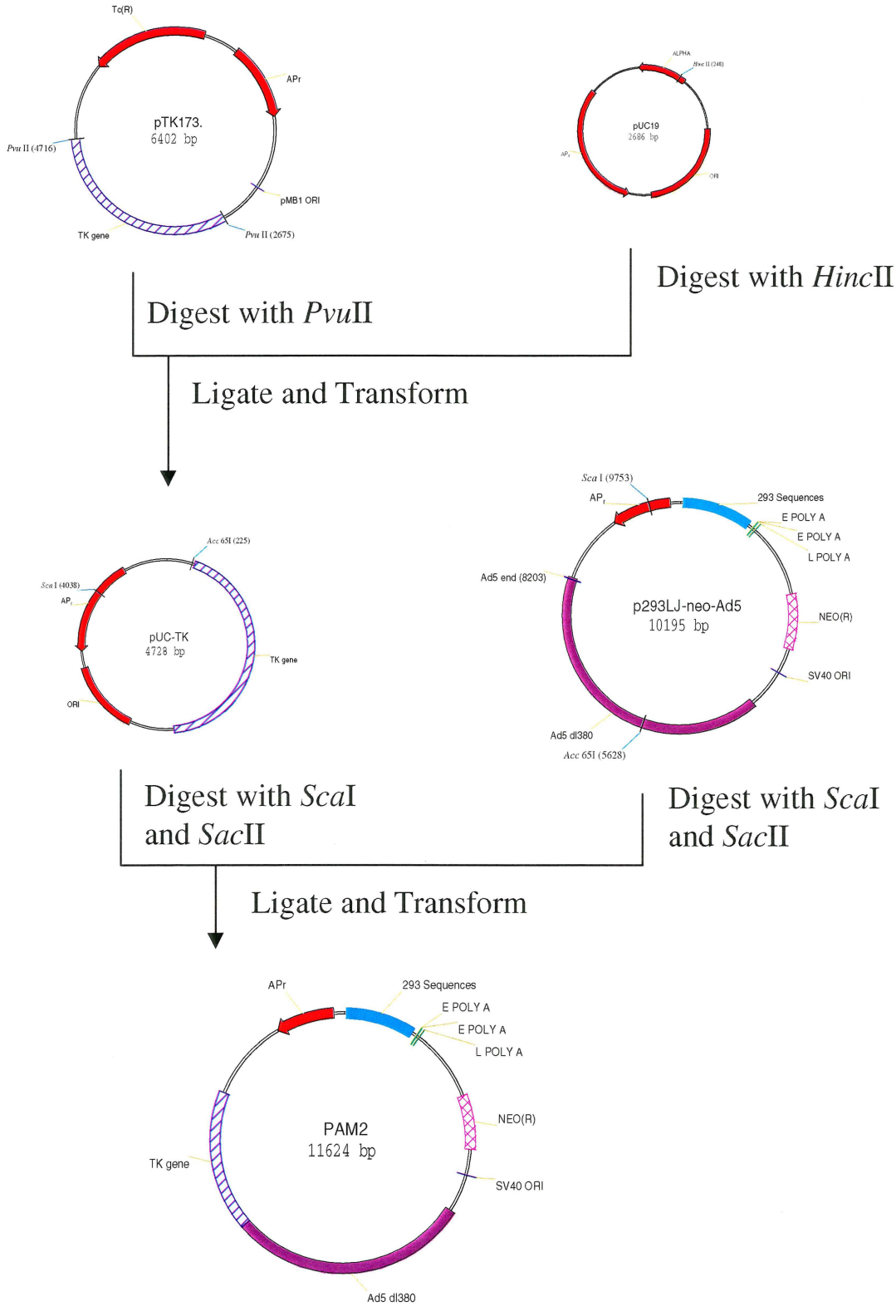
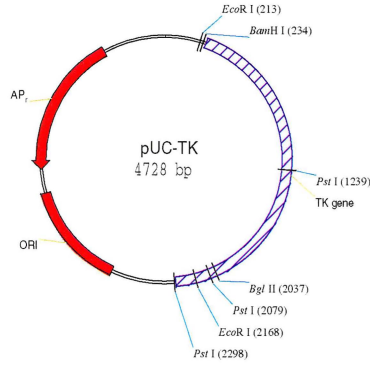


Figure 27. Diagram of the strategy to clone pUC-TK and PAM2 (see text for details).

A



B

Restriction Enzyme	*Eco* RI	*Pst* I	*Bam* HI + *Bgl* II
Fragment Size (bp)	2773 1955	3669 840 219	2925 1803

C

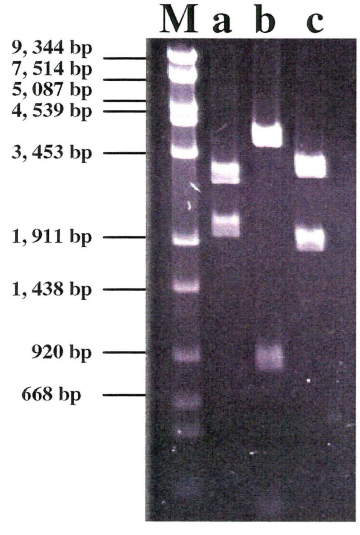


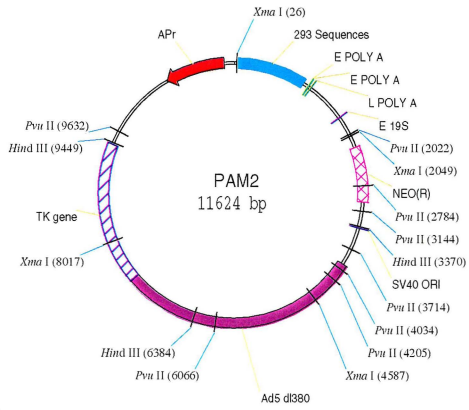
Figure 28. Confirmation of pUC-TK. Panel **A** is a restriction map of pUC-TK showing the relevant sites of *EcoR*I, *Pst*I, *Bam*HI and *Bgl*II. Panel **B** is a table showing the expected fragment sizes when the plasmid is digested with the specified enzymes. Panel **C** is a picture of the restriction fragments of pUC-TK. The M denotes the Norgen N1 marker, lane a is the *EcoR*I digest, lane b is the *Pst*I digest, and lane c is the *Bam*HI and *Bgl*II digest.

3.2.8 CONSTRUCTION OF PAM2

In order to construct the final cassette PAM2, the *tk* gene from pUC-TK needed to be cloned into the plasmid p293LJ-neo-Ad5. This was a relatively straight forward procedure, considering that both of these plasmids are pUC-based (Figure 27). First, p293LJ-neo-Ad5 was digested with *Sca*I and *Sac*II, and the resulting two fragments were run on a gel. The larger fragment, containing the cellular sequences, the *neo* gene and the adenovirus sequences, was then purified from the gel. Next, pUC-TK was also digested with *Sca*I and *Sac*II and the fragments were run on a gel. The larger fragment, containing the *tk* gene was also purified from the gel. The two isolated bands were then ligated together, and resulted in the plasmid PAM2. The plasmid was verified through restriction enzyme analysis (Figure 29).

This final construct now contains: (1) 1001 bps of the cellular-viral junction from 293 cells, (2) the *neo* gene under contol of the SV40 promoter, with an SV40 polyadenlyation signal, (3) the adenoviral sequences from bp 381 to bp 3834, (4) and the *tk* gene under control of its own promoter and polyadenlyation sequence. This cassette can now be used to modify 293 cells through the process of homologous recombination, in order to generate the new cell line of interest (Figure 30). This new cell line will be identical to current 293 cells, except that the first 380 bp of the adenoviral genome will be removed and will be replaced with the *neo* gene. This 380 bp deletion will eliminate the sequences in the cell line homologous to the left-hand side of the transgene in E1-deleted adenoviral vectors, and will thus eliminate the possibility for double crossovers and the generation of RCA.

A



B

Restriction Enzyme	*Hind* III	*Pvu* II	*Xma* I
Fragment Size (bp)	5545	4014	3633
	3065	3566	3430
	3014	1861	2538
		762	2023
		570	
		360	
		320	
		171	

C

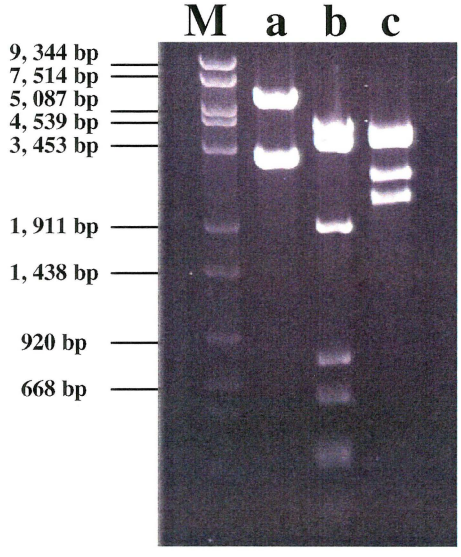


Figure 29. Confirmation of PAM2. Panel **A** is a restriction map of PAM2 showing the relevant sites of *Hind*III, *Pvu*II, and *Xma*I. Panel **B** is a table showing the expected fragment sizes when the plasmid is digested with the specified enzymes. Panel **C** is a picture of the restriction fragments of PAM2. The M denotes the Norgen N1 marker, lane a is the *Hind*III digest, lane b is the *Pvu*II digest, and lane c is the *Xma*I digest.

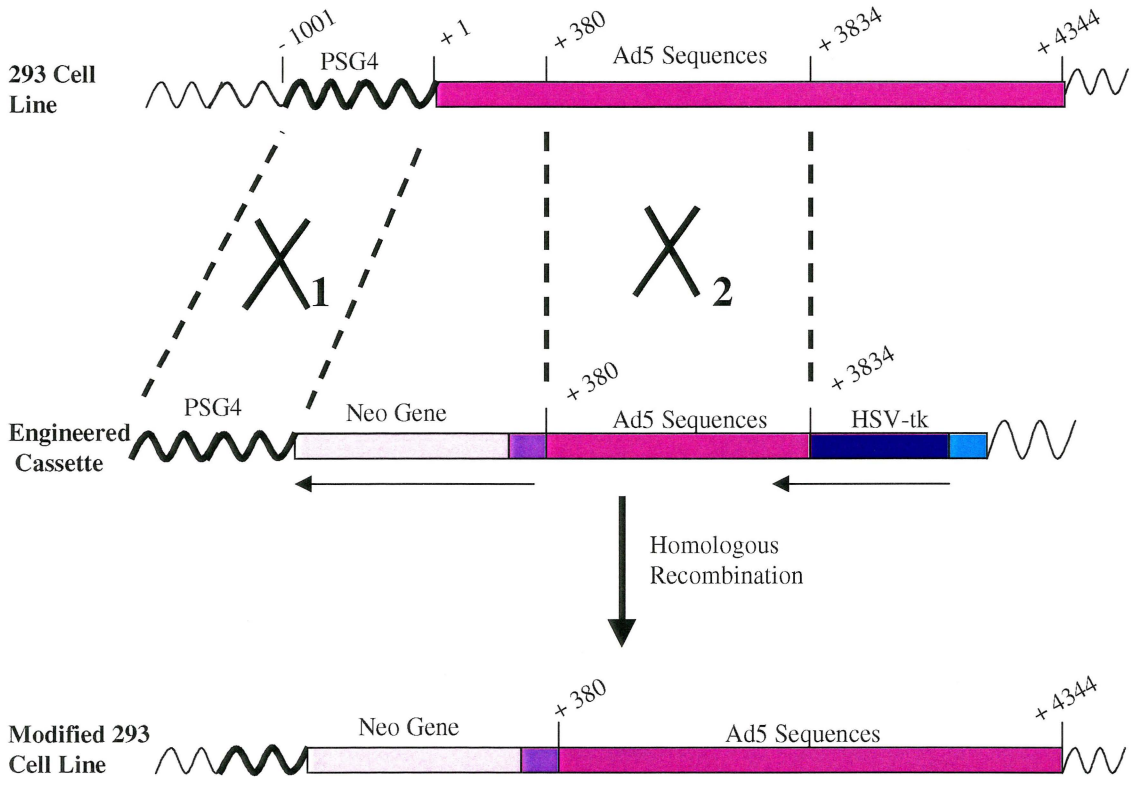


Figure 30. Generation of the modified 293 cell line through homologous recombination between the engineered cassette and the 293 cell line.

3.3 ESTABLISHMENT OF THE MODIFIED 293 CELL LINE

Once the cassette had been engineered, the next step was to transfect the 293 cells with the casette in order to generate the new cell line. When DNA is introduced into mammalian cells, it integrates randomly over the genome by 2-5 orders of magnitude more frequently than homologous recombination occurs (Vega, 1991). Thus, the cassette was designed such that positive/negative selection could be used. This procedure can be used to enrich by 2000-fold for those cells that have taken up the construct through homologous recombination (Mansour *et al.*, 1988).

The cassette contained a *neo* gene for positive selection, such that all cells that have taken up the construct through either homologous or nonhomologous recombination will be resistant to the drug G418. The cassette also contained the HSV-*tk* gene adjacent to one of the regions of homology. In this case, homologous recombination will result in the HSV-*tk* gene not being transferred into the 293 cells. The HSV-*tk* gene is lost during the process of homologous recombination because it is located distal to the region of homology between the vector and the target in the cells. Only cells in which random integration of the targeting vector occurred will be able to retain the HSV-*tk*. Therefore, by using G418 to select for cells that contain a functional *neo* gene and by using gancyclovir to select against cells that have a functional HSV-*tk* gene, one can enrich for cells that have undergone the expected double crossover.

3.3.1 DETERMINATION OF THE LEVEL OF G418 TO BE USED FOR SELECTION

Cell lines differ widely in their sensitivity to G418, and thus, the concentration to be used for selecting stably transfected cells had to be determined experimentally. As described in Materials and Methods, non-transfected cells were subjected to varying levels of G418 during a two-week period to determine the lowest level that would bring about total cell death in that

period. The results of two trials are summarized in Tables 3 and 4. From the results, it was determined that 150-200 μg/ml of G418 would be the ideal concentration to use to select stably transfected 293 cells.

Table 3. Results of varying concentration of G418 on Plate 1 of 293 cells over a two-week period (percentages are estimates of the number of surviving cells, where 100% is a fully confluent plate).

G418 Concentration	Plate 1 t = 0 weeks	Plate 1 t = 1 week	Plate 1 t = 1.5 weeks	Plate 1 t = 2 weeks
50 ug/ml	100%	100%	75%	80%
100 ugml	100%	90%	35%	20%
150 ug/ml	100%	55%	5%	5%
200 ug/ml	100%	20%	0%	0%
250 ug/ml	100%	5%	0%	0%
control	100%	0%	0%	0%

Table 4. Results of varying concentration of G418 on Plate 2 of 293 cells over a two-week period (percentages are estimates of the number of surviving cells, where 100% is a fully confluent plate).

G418 Concentration	Plate 2 t = 0 weeks	Plate 2 t = 1 week	Plate 2 t = 1.5 weeks	Plate 2 t = 2 weeks
50 ug/ml	100%	100%	70%	50%
100 ugml	100%	90%	40%	10%
150 ug/ml	100%	60%	5%	0%
200 ug/ml	100%	20%	0%	0%
250 ug/ml	100%	5%	0%	0%
control	100%	0%	0%	0%

3.3.2 DETERMINATION OF THE LEVEL OF GANCYCLOVIR TO BE USED FOR SELECTION

The HSV-*tk* gene causes susceptibility to the non-toxic pro-drugs gancyclovir and acyclovir. However, it has been previously determined that gancyclovir is a better choice to use for selection in targeting experiments (Mansour *et al.*, 1988). The level of GANC to be used in these transfections was based on literature values and was 2 x 10^{-6} M (Mansour *et al.*, 1988). Although it has been previously shown that neither the morphology or viability of 293 cells is affected by GANC up to concentrations of 80 μM (Imler *et al.*, 1995), the experimental level of GANC needed to be tested on the 293 cells to be used for transfection. Thus, as described in the Materials and Methods, 293 cells were grown for two weeks in media supplemented with 2 x 10^{-6} M GANC and were compared to 293 cells that were grown in regular media. After the two week period, the cells grown in GANC were morphologically identical to the control cells, and no cell death had occurred (Figure 31). Thus, this was an acceptable level of GANC to use for selection of transformants.

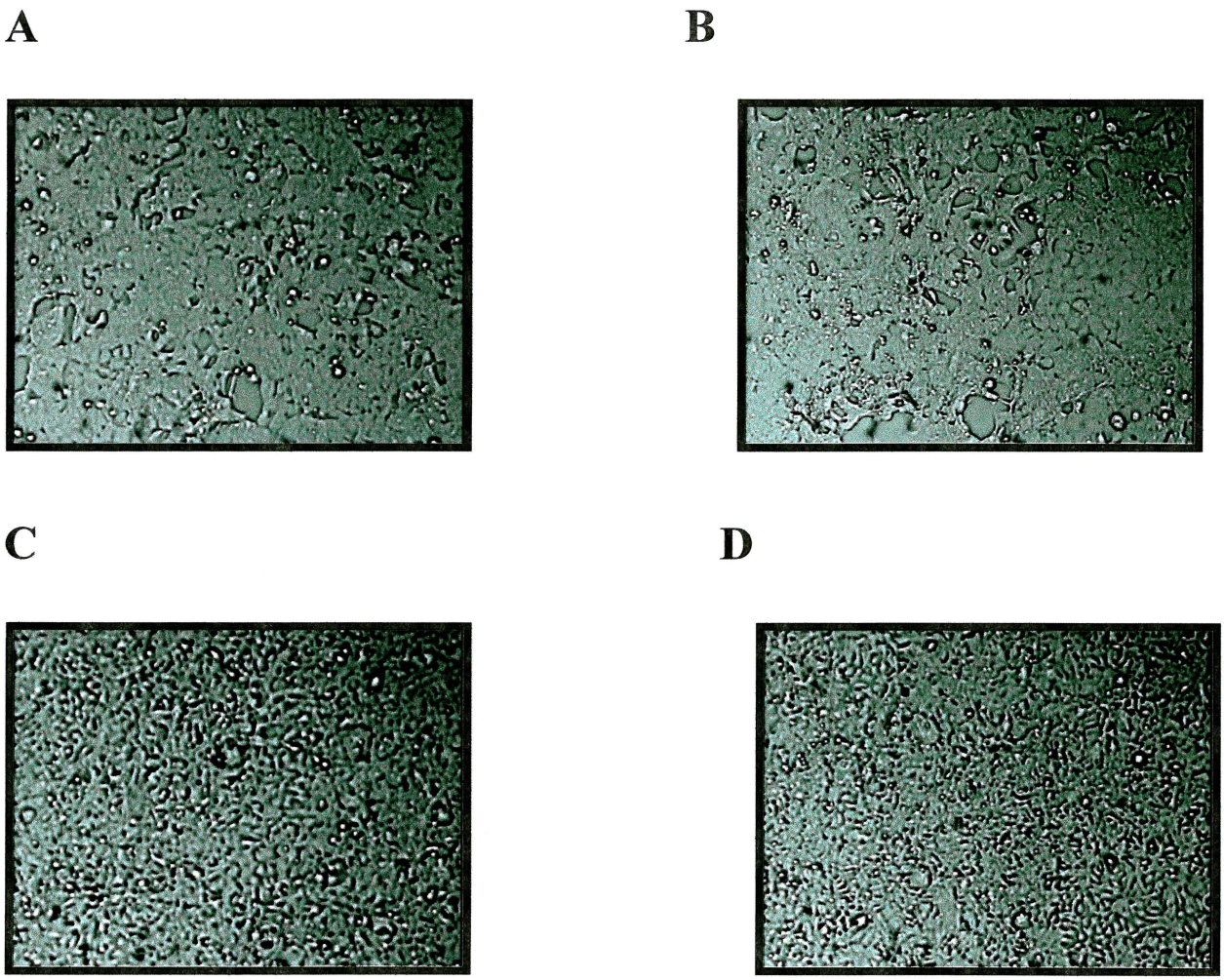


Figure 31. Effect of GANC on 293 cells. Panels **A** and **C** show 293 cells that have been exposed to 2 x 10^{-6} M GANC for 1 week and 2 weeks respectively, and panels **B** and **D** are control 293 cells after 1 and 2 weeks respectively. Magnification is 100X.

3.3.3 CALCIUM PHOSPHATE TRANSFECTION OF 293 CELLS

In order to establish the modified 293 cell line, the PAM2 construct was transfected into 293 cells. The calcium phosphate technique (Graham *et al.*, 1973) was employed, as described in Materials and Methods, and a total of 17 tranfections were performed. Typically, either 2 µg or 5µg of *Nde*I linearized vector was used and 5 x 10^6 cells were transfected. Linearization of the vector outside of the region of homology has been shown to increase the frequency of homologous recombination (Smithies *et al.*, 1985; Hasty *et al.*, 1991; Yanez and Porter, 1998). Selection for cells that had integrated the construct either randomly or through homologous recombination began 2 days after transfection and involved the use of 150-200 µg/ml of G418. Selection proceeded for approximately 2 weeks, or until all the control cells for a given experiment had been killed (Figure 32). Next, selection began on the $G418^r$ pools in order to eliminate the cells that had undergone nonhomologous recombination. Selection with GANC proceeded for approximately 1½-2 weeks, until all the GANC sensitive colonies had been destroyed, resulting in a number of $G418^r$ + $GANC^r$ colonies that could be picked and expanded for screening (Figure 33).

The promising foci were picked using cloning rings and were transferred to 24-well plates. The cells were expanded and then split, with one plate being maintained in the incubator while genomic DNA was extracted from the other plate. A total of 100 pools of $G418^r$ + $GANC^r$ foci were picked for screening.

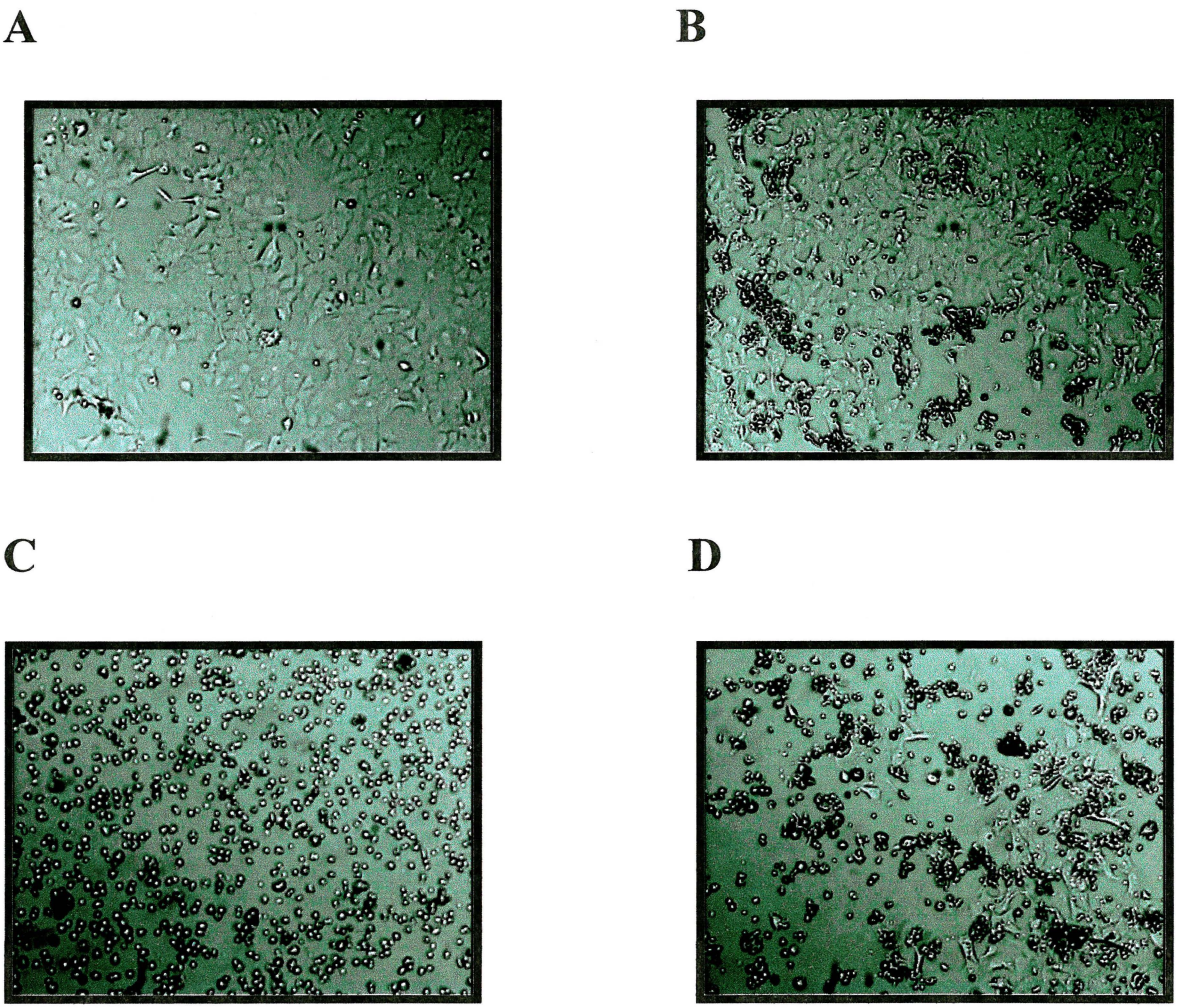
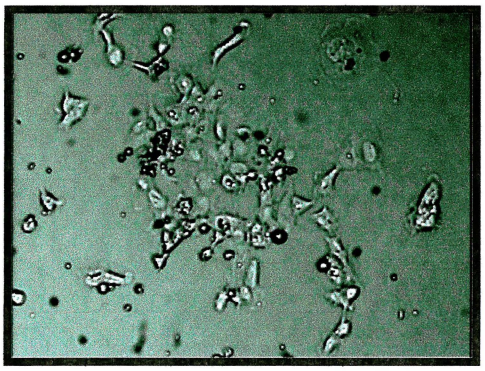


Figure 32. Transfection and selection of 293 cells. Panel **A** shows 293 cells prior to transfection, and panel **B** shows 293 cells after 8 days of selection in 200 μg/ml G418, with some cell death beginning. Panel **C** shows total cell death in control 293 cells after 16 days selection in 200 μg/ml G418, and panel **D** shows some $G418^r$ transfected cells after 16 days selection in 200 μg/ml G418. Magnification is 100X.

A



B

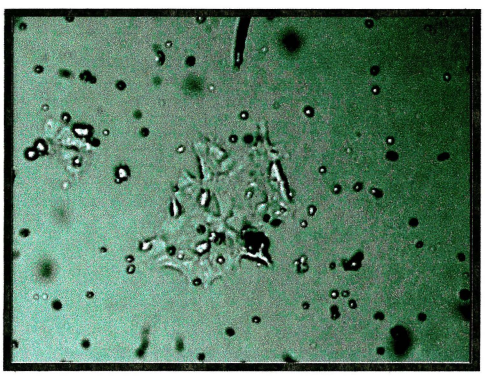


Figure 33. Examples of G418^r and GANC^r foci. Panels **A** and **B** depict foci that were present after 16 days selection in 200 μg/ml G418 and 8 days selection in G418 + 2 x 10^{-6} M GANC. Magnification is 100X.

3.3.4 SCREENING OF G418^r + GANC^r POOLS

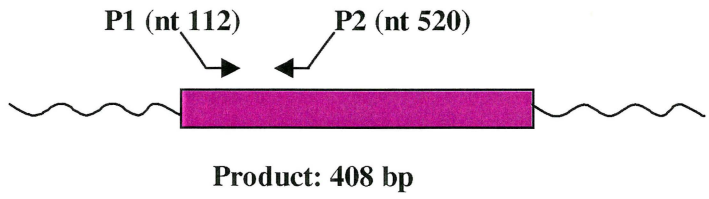
PCR was used in order to screen the cell lines, to determine if the construct had been integrated into the 293 genome via homologous or non-homologous recombination. One set of primers was generated to screen specifically for the desired 380-bp deletion. Primer 1 was designed to anneal from nt 112 to nt 136 of the adenoviral genome, while primer 2 annealed from nt 496 to nt 520. If the construct had integrated through non-homologous recombination, then a 408 bp product would be generated. However, if the cell line had undergone homologous recombination with the vector, the first 380 bp of the adenoviral genome would be deleted, and thus, there would be no PCR product generated (Figure 34).

The absence of a PCR product is not a definitive test, as the PCR may fail for numerous reasons. Therefore, a second set of primers was generated to be used as positive control. Primer 3 annealed from nt 1021 to nt 1045 of the adenoviral genome, and primer 4 annealed from nt 1511 to nt 1535. Since this region is located outside of the 380 bps that were to be deleted, then this primer pair should generate a 514 bp product regardless of whether the cells have undergone homologous or non-homologous recombination (Figure 35).

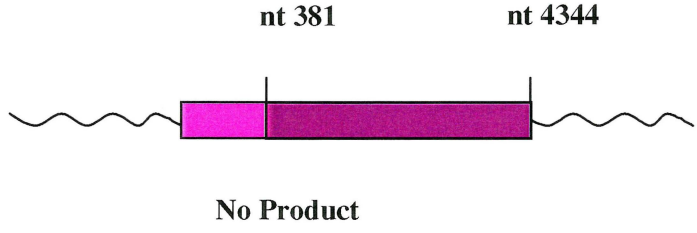
The 100 isolated pools of G418^r + GANC^r cells were then screened using the first primer pair. The absence of a product was promising for the 380 bp deletion, and the PCR would be repeated in these cases (Figures 36 and 37). If there was still no formation of a PCR product, then more genomic DNA would be isolated from these cells and the PCR would be performed again using both the test primers and the control primers. In many cases, cells which had initially been giving a negative signal for the 380 bp region were now showing amplification products using the first primer pair (Figure 38). Possible reasons for this are explored in the Discussion section. However, ten pools of G418^r + GANC^r cells were deemed to be promising and, therefore, needed to be subcloned in order to isolate homogenous cell lines. This was done

through single-cell cloning by the end-point dilution technique, as described in the Materials and Methods.

A 293 Cells



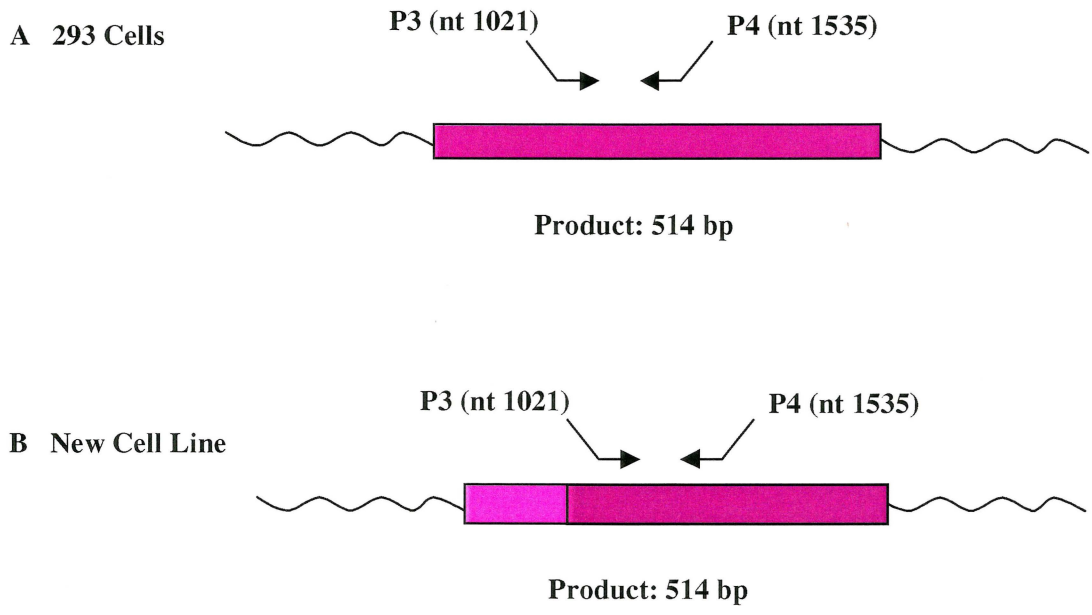
B New Cell Line



C

Primer	Sequence of Primer (5' to 3')	Location of Primer*
P1: Ad5 - For	CGGAAGTGTGATGTTGCAAGTGTGG	nt 112 of Ad5
P2: Ad5 - Rev	CTACTCGCTGGCACTCAAGAGTGG	nt 520 of Ad5

Figure 34. Strategy for the screening of homologous recombinants by PCR amplification. Panel **A** shows that a 408 bp product will be generated if the cell line has undergone non-homologous recombination, and panel **B** shows that no product will be formed if the cells have undergone homologous recombination with the construct. Panel **C** shows the sequence of the primers as well as their exact location in terms of the 293 genome (* position of the 5′ nucleotide).

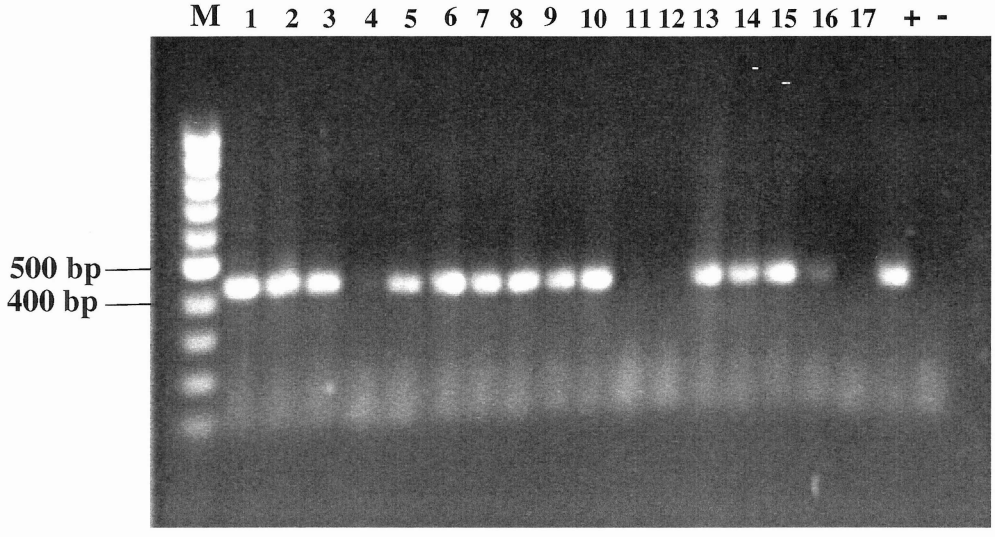


C

Primer	Sequence of Primer (5' to 3')	Location of Primer*
P1: Ad5 - ForCon	CAGGTCTTGTCATTATCACCGGAGG	nt 1021 of Ad5
P2: Ad5 - RevCon	GGCGTTTACAGCTACAGTCCAAAGG	nt 1535 of Ad5

Figure 35. Diagram of the positive control to be used in the screening process for homologous recombinants. Panels **A** and **B** show that a 514 bp product will be formed if the cells have undergone homologous recombination or non-homologous recombination with the construct. Panel **C** shows the sequence of the primers as well as their exact location in terms of the 293 genome (* position of the 5′ nucleotide).

A



B

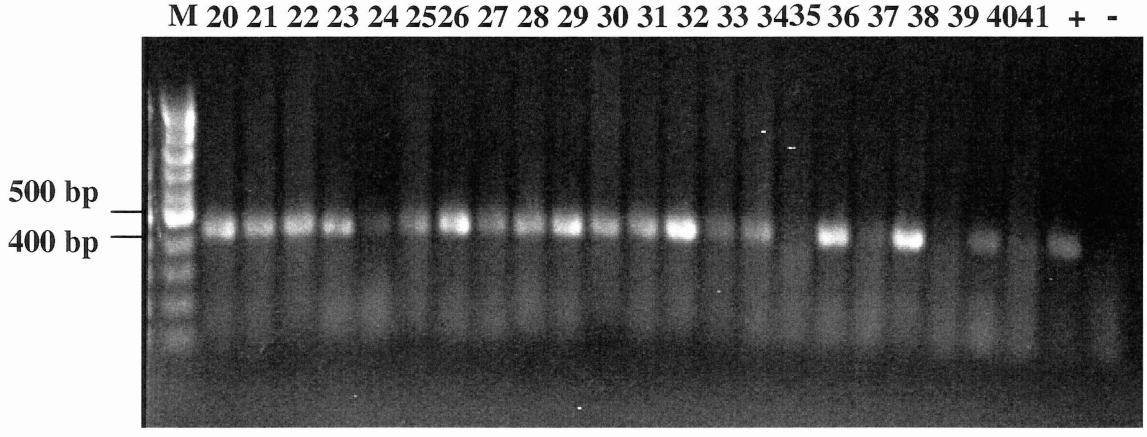


Figure 36. Examples of agarose gel pictures of the first round of PCR screening for homologous recombinants. M denotes the Norgen PCR-Sizer marker, + is the positive control of 293 genomic DNA, and – is the negative control of no DNA. The numbers indicate different genomic DNA samples. In panel **A**, samples 4, 11, 12 and 17 show negative signals, while in panel **B**, samples 24, 35, 37, 39 and 41 exhibit negative signals.

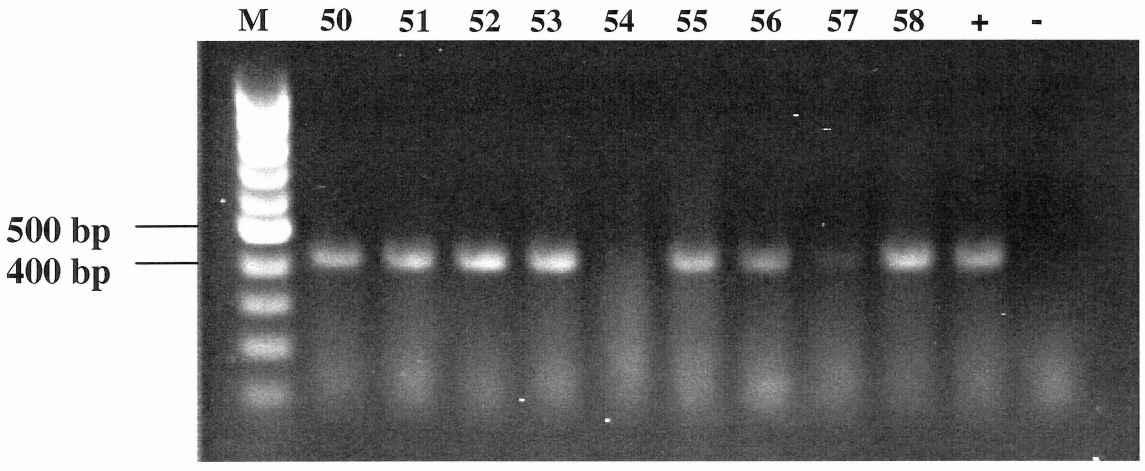


Figure 37. Example of an agarose gel picture of the first round of PCR screening for homologous recombinants. M denotes the Norgen PCR-Sizer marker, + is the positive control of 293 genomic DNA, and – is the negative control of no DNA. The numbers indicate different genomic DNA samples. Samples 54 and 57 show negative signals for the 380 bps.

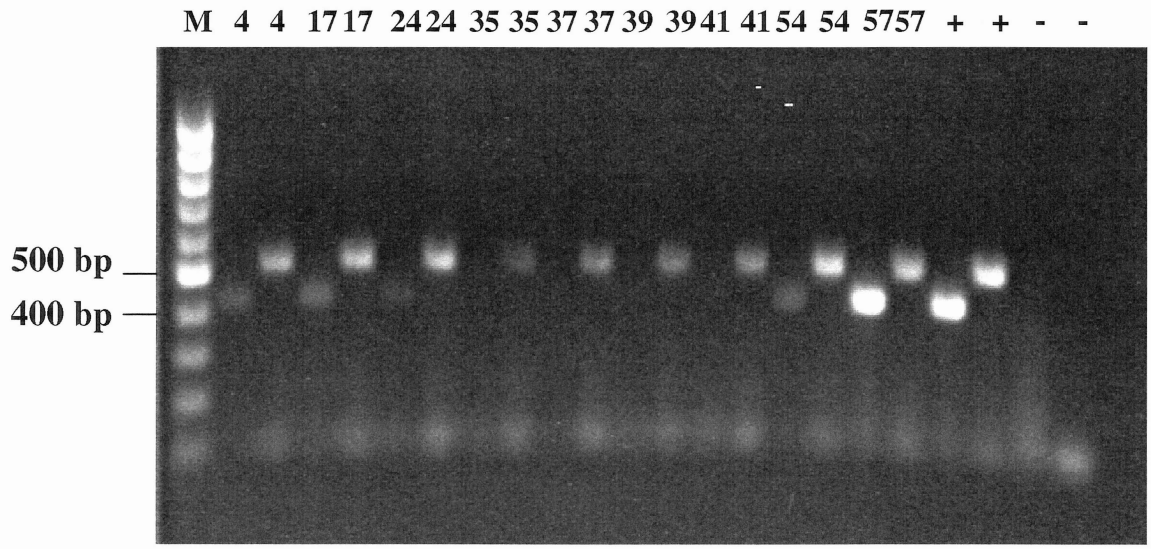
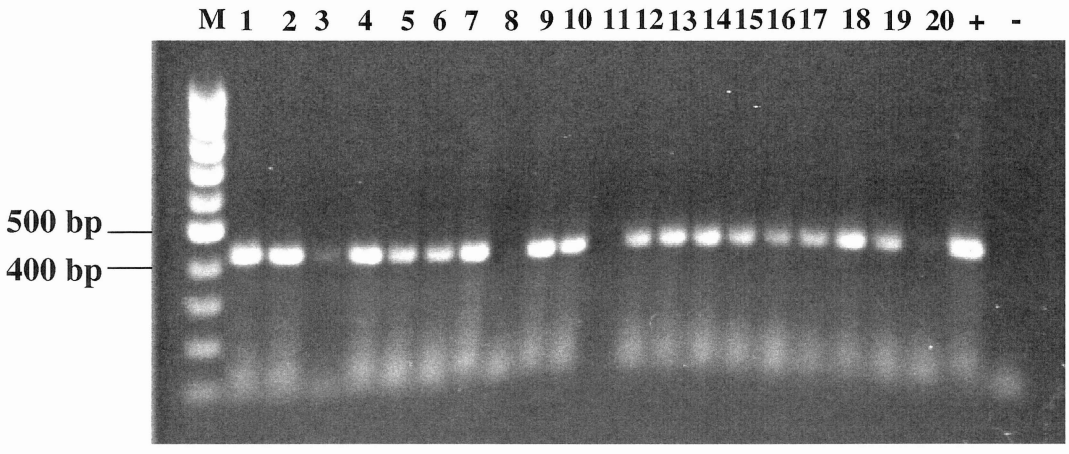


Figure 38. Example of an agarose gel picture of the second round of PCR screening for homologous recombinants. M denotes the Norgen PCR-Sizer marker, + is the positive control of 293 genomic DNA, and – is the negative control of no DNA. The numbers indicate different genomic DNA samples. Samples 4, 17, 54 and 57 are now showing positive signals for the 380 bp region, while samples 24, 35, 37, 39 and 41 look promising for the deletion of the 380 bp region.

3.3.5 SCREENING OF G418^r + GANC^r FOCI

As stated, the cells from the ten most promising pools were diluted to single cells, and plated in large plates at a concentration of 50 cells/plate such that individual, homogeneous foci could grow. Ten foci from each of these plates were then picked using cloning rings and again transferred to 24-well plates to allow for expansion. Genomic DNA was isolated from these cells, and the same process of PCR screening for detection of homologous recombinants was employed (Figure 39). From the initial screening, eight samples looked promising for the deletion of the 380 bp and thus homologous recombination. Again, the PCR was repeated and additional genomic DNA was extracted from the cells. Upon performing the final PCR in which both primer pairs were used, all the samples showed a positive signal for the 380 bps (Figure 40). Possible reasons for these results are described in the Discussion section.

A



B

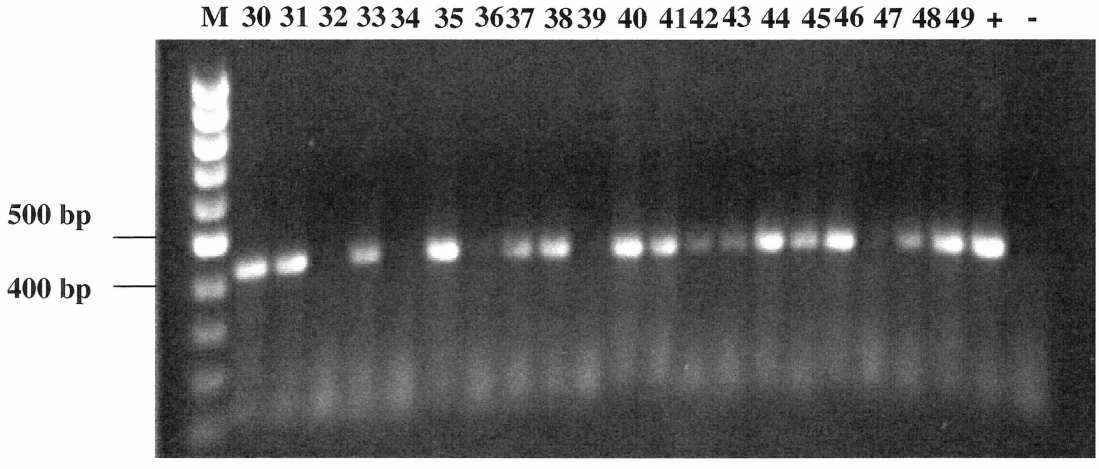


Figure 39. Examples of agarose gel pictures of the first round of PCR screening of homogeneous foci for homologous recombinants. M denotes the Norgen PCR-Sizer marker, + is the positive control of 293 genomic DNA, and – is the negative control of no DNA. The numbers indicate different genomic DNA samples. In panel **A**, samples 8, 11, and 20 show negative signals, while in panel **B**, samples 32, 34, 36, 39 and 47 exhibit negative signals.

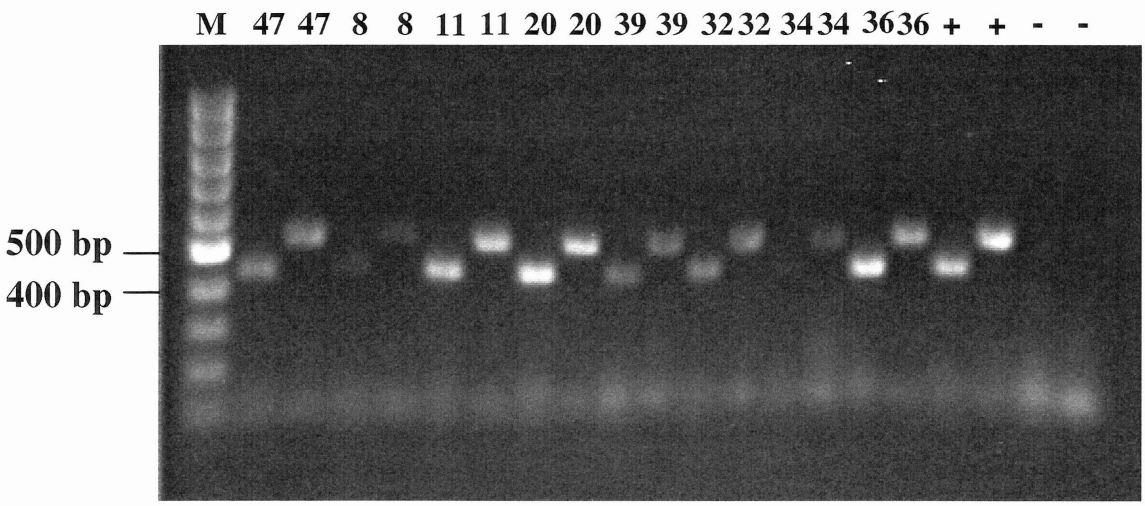


Figure 40. Example of an agarose gel picture of the second round of PCR screening of homogeneous foci for homologous recombinants. M denotes the Norgen PCR-Sizer marker, + is the positive control of 293 genomic DNA, and – is the negative control of no DNA. The numbers indicate different genomic DNA samples. All samples show a positive signal for the presence of the 380 bp region.

4. DISCUSSION

The generation of replication competent adenoviruses (RCA) is a major problem that faces the use of recombinant adenovirus vectors for gene therapy. The presence of RCA in Ad vector batches that are to be used clinically is undesirable and constitutes a major safety risk. The main concern is that replication competent vectors may provide helper functions to the replication-defective vectors, and may lead to uncontrolled amplification and spread of the replication-defective vector viruses (Imler *et al.*, 1995). Furthermore, the RCA may result in aggravation of the host immune response to give rise to inflammation and tissue damage (Fallaux *et al.*, 1998). As a result of these potential problems, regulatory bodies such as the Food and Drug Administration (FDA) of the United States have taken the position that RCA are unacceptable in adenoviral vector preparations for clinical use (Fallaux *et al.*, 1998).

Although the FDA takes this position, strict guidelines have not yet been established as to the level of RCA that is acceptable in vector preparations, nor is there a standardized test for RCA. There are inconsistencies in what analytical methods are used to quantitate RCA, and RCA testing is not consistent among different manufacturers (www.fda.gov/cber/minutes/aden020101.htm). Therefore the FDA has taken a conservative approach and assumed that there is a safety risk associated with RCA; however, they have not determined what level is actually safe. Furthermore, the FDA suggests that a cell culture/cytopathic effect method be used to test for RCA, but they do not indicate input multiplicity of infection (MOI), what cells to perform the test on, or what volume of production lot should be tested (www.fda.gov/cber/somgene.txt). The FDA also suggests that the assay be quantitative; however, in application the test is usually semi-quantitative and involves a yes or no answer (www.fda.gov/default.htm). All these factors lead to huge inconsistencies in RCA

testing, and potential dangers for patients involved in Ad gene therapy trials. The FDA is currently working on developing an adenoviral standard that would deal with not only the issue of RCA, but also various other issues facing the use of adenoviral vectors in clinical trials such as virus generation and dose levels (www.fda.gov/cber/minutes/aden020101.htm).

Studies have shown conclusively that RCA are generated by homologous recombination between vectors and 293 cell Ad5 sequences (Hehir *et al.*, 1996; Zhu *et al.*, 1999). It has been determined that 293 cells contain the Ad5 genome from the terminal left-end to nucleotide position 4344 (Louis *et al.*, 1997). There are various ways to make E1 deletion vectors; however, the most common ones result in at least 350-450 nucleotides of homologous sequence on the left side of the transgene and about 800 bp of homology on the right of the transgene (Hehir *et al.*, 1996). Analysis of RCA isolates indicates that dual recombination events to the left and right of the transgene result in the loss of the transgene and acquirement by the vector of the E1 region (Hehir *et al.*, 1996). One strategy to achieve RCA-free Ad preparations is to remove the homologous sequences from either side of the transgene in order to eliminate the possibility of homologous recombination through a double crossover.

Many different groups of researchers have attempted to overcome the problem of RCA generation in 293 cells. Traditionally, this has involved attempts to establish new packaging cell lines or to modify the adenoviral vector backbone. Hehir and colleagues found a reduction in the rate of RCA formation in 293 cells through the deletion or rearrangement of pIX gene sequences within the adenoviral vectors, but this strategy did not completely eliminate RCA formation (Hehir *et al.*, 1996). Imler *et al.* isolated an A549 cell line transfected with a plasmid containing E1A and E1B open reading frames with the contiguous pIX (bp 505 to 4034), in which E1A is driven by a phosphoglycerate kinase (PGK) promoter. This cell line does not generate RCA from a vector deleted of only E1A and E1B (bp 104 to 3329) (Imler *et al.*, 1996). Another group has developed a new packaging cell line and matched adenoviral vectors (Fallaux *et al.*, 1998).

The PER cells produced by Fallaux *et al.* contain the Ad5 genome from nucleotides 459-3510 under control of the PGK promoter, and the matched Ad vectors do not have any sequence overlap. Propagation of the matched Ad vectors in the PER cells does not result in the generation of RCA. Furthermore, the cell line has no sequence overlap between integrated adenoviral DNA and vector DNA of commonly used vectors. Schniedner and colleagues (2000) have established adenovirus packaging cell lines by transfecting primary human amniocytes with the Ad5 E1 region. The generation of RCA was excluded in these cell lines by designing the transforming plasmid to lack the first 505 bp of the adenoviral genome, and thus sequence overlap with current adenoviral vectors on the left-hand side of the transgene. Gao *et al.* (2000) have developed a HeLa-based cell line, GH329, which contains the Ad5 E1 region from bp 511 to 3924 under control of the PGK promoter. There is no overlap with the 5′ region of standard E1-deleted vectors and reduced overlap at the 3′ end. Again, this cell line does not result in the formation of RCA upon serial passaging of an E1-deleted virus (Gao *et al.*, 2000). Lastly, Kim *et al.* (2001) have also developed a HeLa-based packaging cell line, that contains and expresses the minimal E1 gene. This HeLa-E1 cell line was shown to produce no RCA during a minimal number of passages of an E1-deleted vector (Kim *et al.*, 2001). Thus a number of researchers have overcome the problem of RCA formation through the generation of new packaging cell lines.

As mentioned earlier, there is no standardized test for the presence of RCA, and there are numerous variations in testing. For example, Fallaux *et al.* (1998) performed a detailed cytopathic effect (CPE) assay involving 2 cell lines to test for RCA. The assay involved infecting HeLa cells with the virus at an MOI of <25, waiting 4 days and then passing a freeze-thaw lysate of the HeLa cell culture assay on to cultures of A549 cells. The observation of either individual plaques or widespread CPE was taken as an indication of the presence of RCA in the initial dose (Fallaux *et al.*, 2001). Gao *et al.* (2001) also used a CPE assay to test for the

generation of RCA. However, in their case 1 x 10^8 PFU of virus was added to A549 cells, and infected cells from each plate were harvested and lysed 14 days later. The lysate was applied to A549 cells, and after 7 days the plates were examined for CPE. It is difficult to directly compare the results from these two groups due to the fact that the initial amount of virus tested was measured in different ways, and the amount and type of cells being used for the assay are different. Perhaps RCA may have been detected by one of the groups if they had used the other testing method. A completely different method used to screen for RCA was PCR (Kim *et al.* 2001). The PCR was based on detecting the presence of the E1A gene in the DNA isolated from virus. This method of RCA detection is very different from the CPE assays used by the other two groups, and again it is difficult to compare results because of these inconsistencies. Furthermore, Kim *et al* (2001) tested for the presence of RCA after only two passages through the cell line, while Gao *et al.* (2000) tested for RCA after 20 passages. It is important to keep these ideas in mind when considering the value of a new packaging cell line in terms of RCA formation.

In this research, it was decided that instead of following the traditional methods of generating a new packaging cell line to eliminate the generation of RCA, the 293 cell line itself would be modified. The first 380 bps of the adenoviral genome would be removed from the cells, and therefore the regions of homology on the left-hand side of most commonly used E1-deleted vectors would be removed. Thus, there would be no possibility of a double-crossover event occurring, and the generation of RCA would be eliminated.

The method that was used to modify the 293 cell line was the process of homologous recombination, or gene targeting. Gene targeting is essentially the homologous recombination of DNA sequences residing in the chromosome with newly introduced DNA sequences, and it provides a means for systemically altering the mammalian genome (Jasin *et al.*, 1985; Smithies *et al.*, 1985; Thomas *et al.*, 1986, Thomas and Capecchi, 1987). The main drawback for gene

targeting is that it is quite inefficient, and typically the absolute efficiency ranges from 10^{-5} to 10^{-7} targeted cells per transfected cell (Yanez and Porter, 1998). Furthermore, when DNA is introduced into mammalian cells, it integrates randomly over the genome by 2-5 orders of magnitude more frequently than homologous recombination occurs (Vega, 1991). The ratio of homologous to non-homologous recombination in mammalian cells is about 1:1,000 (Capecchi, 1990). Researchers have been very interested in determining ways to increase the frequency of homologous recombination, as well as developing methods that can help to screen for homologous recombinants.

Many points were taken into consideration when designing the modification cassette PAM2. Firstly, it has been determined that one of the key factors in efficient homologous recombination is the use of isogenic DNA to prepare the vector (Riele *et al.*, 1992). On average, vectors prepared from isogenic DNA are four to five times more efficient than corresponding vectors prepared from nonisogenic DNA (Deng and Capecchi, 1992). Therefore, in this project the cellular-viral junction was cloned from genomic DNA that was isolated from the 293 cell line that would ultimately be used for transfections.

Secondly, the cassette was designed such that the enrichment procedure of positive-negative selection (PNS) could be employed (Mansour *et al.*, 1988; Capecchi, 1989). Essentially, this procedure uses a positive selection for cells that have incorporated the targeting construct anywhere in the genome, and a negative selection against cells that have randomly integrated the vector. The vector is designed to carry the left-hand region of homology, a *neo* gene, the right-hand region of homology, and the HSV-*tk* gene. This vector was designed such that homologous recombination will result in the HSV-*tk* gene not being transferred into the 293 cell line. The HSV-*tk* gene is lost because it is located distal to the right-hand side region of homology, and only cells in which random integration of the targeting vector has occurred will be able to retain the HSV-*tk* gene. Therefore, by selecting for cells that contain a functional *neo*

gene and by using GANC to select against cells that contain a functional HSV-*tk* gene, one can enrich for cells in which the targeting event has occurred. This procedure has been found to enrich 2,000-fold for cells that contain a targeted alteration (Mansour *et al.*, 1988).

Lastly, it was important to construct the cassette such that it had a sufficient amount of homology to allow for homologous recombination. It is widely accepted that recombination frequency decreases as the length of homology is reduced (Rubnitz and Subramani, 1984; Hasty *et al.*, 1991; Deng and Capecchi, 1992). There is some discrepancy regarding the minimum amount of homology required, and whether it is the total amount of homology present that has the greatest effect on targeting frequency, or the length of homology on the short arm. Rubnitz and Subramani (1984) have reported very low recombination frequencies with as little as 14 base pairs of homology (Rubnitz and Subramani, 1984). Hasty *et al.* (1991) determined that 472 bp of homology on one arm is used as efficiently as 1.2 kb in the formation and resolution of cross-over junctions. Furthermore, they determined that the total length of homology has a greater impact on targeting efficiency than the length of homology on the short arm (Hasty *et al.*, 1991). Contrary to these findings, Thomas *et al.* (1992) found that it is a reduction in arm length rather than a reduction in the total amount of absolute homology that led to inaccurate recombination. They determined that if one arm of the construct contains less than 1 kb of homologous DNA, abortive recombination can occur (Thomas *et al.*, 1992). In the present study, the construct PAM2 contained 1001 bps of DNA that spans a region that is homologous to the cell line on the short arm, and 3454 bp of homologous DNA on the long arm. Therefore, the total amount of homology is 4455 bp, which is deemed sufficient for recombination.

The first task of this project was to clone the cellular-viral junction from 293 cells, which would serve as one arm of homology in the cassette. PCR amplification was used to clone the junction, and the design and location of the primers are described in Section 3.1 of Results. The primer design was based on the work of Louis *et al.* (1997), who determined that the Ad5

sequences in 293 cells are integrated into chromosome 19, at the PSG4 gene. The plasmid p293LJ was constructed and sequenced, and the sequence was analyzed using BLAST (Altschul *et al.*, 1997). Upon alignment of the p293LJ sequence and chromosome 19, it was found that the 293 cell line used in this work appears to contain a 3928-bp deletion. The p293LJ sequence aligns with chromosome 19 from the point of Ad5 integration back for 176 base pairs, and then aligns from –4104 to –4871 on chromosome 19, using the site of Ad integration as 0. Therefore, it appears that there is a 3928 bp deletion from –176 to –4104 (Figure 11). It should be noted that low passage number 293 cells were used for this thesis, and that the cells had just recently been purchased prior to the PCR amplification of the cellular-viral junction.

This deletion is not surprising, considering the fact that this is a laboratory cell line that has been grown and passaged since 1977 (Graham *et al.*, 1977). As in any other cells, processes such as insertions, deletions, translocations and recombination are occurring frequently. The main selective pressure on these human embryonic kidney cells is that the E1 region of Ad5 is being expressed. The only other selective pressure on this cell line would be for growth and maintenance functions. The PSG4 gene, into which the Ad5 sequences are integrated, belongs to the human pregnancy-specific β1 glycoprotein family. These are a group of glycoproteins which are synthesized in large amounts by placental trophoblasts. During pregnancy, PSG is released to the maternal circulation and, as pregnancy progresses, an increasing concentration of PSG is found in maternal blood (Khan *et al.*, 1992). Although the PSGs were discovered more than 25 years ago, their function is still unknown (Teglund *et al.*, 1994). Therefore, it does not appear that this PSG4 gene would supply any necessary function for the growth and maintenance of the 293 cells, and that a large deletion in this gene family would not be detrimental to the integrity of the cell line.

The second objective of this project was to construct the gene targeting cassette PAM2. The design and components of the cassette have been discussed previously. All plasmid

intermediates for the construction of the cassette were verified by restriction enzyme analysis and agarose gel electrophoresis.

The third objective was to transfect 293 cells with the constructed cassette. Numerous transfection methods are available for use with mammalian cells including the calcium phosphate technique, electroporation, Lipofectamine, and injection of DNA. In this case, the calcium phosphate procedure was chosen, due to its attractive features including (a) it is a very efficient method to use with 293 cells, (b) it is relatively easy to perform and inexpensive, and (c) it was invented by the same researchers who initially developed the 293 cell line (Graham and van der Eb, 1973). Calcium phosphate transfections have been previously shown to be successful in a number of gene targeting experiments (Smith and Berg, 1984; Lin *et al.*, 1985; Jasin *et al.*, 1985; Jasin and Berg, 1988; Wang *et al.*, 1995; Mathews *et al.*, 1999).

The transfection and selection procedures were performed as described in the Materials and Methods section. Linearization of the targeting construct has been found to increase the frequency of homologous recombination (Smithies *et al.*, 1984; Jasin *et al.*, 1985; Thomas *et al.*, 1986; Yanez and Porter, 1998). Linear DNA molecules appear to be much more recombinogenic in mammalian cells than their corresponding supercoiled molecules (Thomas *et al.*, 1986). It has been found that the location of the linearization site on either the 5′ or 3′ region of homology does not alter the targeting frequency (Hasty *et al.*, 1991). The targeting vector PAM2 was linearized prior to transfection using *Nde*I, which cut the cassette outside of the 5′ region of homology.

A total of 17 transfections were performed. Selection would begin with G418 after two days, and continued for approximately two weeks or until all the control cells were killed for a given experiment. Typically, from the 500,000 cells that were initially transfected, the number of $G418^r$ cells would be too many to count. This would correspond to hundreds of $G418^r$ cells, which is comparable to literature values for other gene targeting experiments (Lin *et al.*, 1985;

Smith and Berg, 1984; Thomas *et al.*, 1986). These $G418^r$ cells represent any cell that has integrated the construct, either through random or homologous recombination. Upon selection with GANC, a large number of the $G418^r$ colonies would be killed, until approximately 3-14 $G418^r$ + $GANC^r$ colonies would remain. Ideally, these colonies would represent only the cells that had taken up the construct through homologous recombination. The cells would then be picked for screening using cloning rings. These cells should be viewed as pools at this point for two reasons. First, it would be incorrect to assume that these colonies arose from a single cell, due to the fact that there were 500,000 cells initially present in these 35mm dishes. Second, when the foci were picked using cloning rings, often more than one focus would be included due to the large size of the cloning rings. Thus, these should be viewed as pools of $G418^r$ and $GANC^r$ cells.

A number of different methods are available to screen for homologous recombination. Initially, genomic Southern blots were the screening method of choice and were used almost exclusively in gene targeting experiments (Thomas and Capecchi, 1986; Hasty *et al.*, 1991; Deng *et al.*, 1993). However, the popularity of Southern blots has recently been diminished with the arrival of PCR-based assays (Kim and Smithies, 1988; Wang and Taylor, 1993; Abrahams *et al.*, 1998). Novel methods for screening have also been developed, including an ELISA-based method (Williams *et al.*, 1994). Considering that PCR had already been used and termed efficient in this study, along with the fact that it is quick and requires extremely small amounts of DNA, PCR was chosen as the screening method for detection of homologous recombinants.

Normally when using PCR to screen for homologous recombinants, the basic strategy is to select primers such that amplification can only occur when homologous recombination has taken place. One primer is chosen from the region unique to the construct and the other primer is chosen from genomic DNA sequences in the chromosome but outside of the region of interest (Frohman and Martin, 1990). In this case it was difficult to design the PCR primers this way,

due to the fact that the inserted *neo* gene and regulatory sequences were so large (~3 kb), and the only difference between the new cell line and the 293 cell line is the presence or absence of the 380 bp region. Thus the screening strategy outlined in the results section was employed, in which two pairs of primers were used. The first pair would result in a product only if the 380 bp were present, and therefore homologous recombination had not occurred (Figure 33). The second primer pair was designed to amplify in the case of both homologous and non-homologous recombination, and thus acted as a positive control (Figure 34).

Genomic DNA was isolated from the $G418^r$ + $GANC^r$ colonies, and was initially screened using the first primer pair. If the result of the PCR was negative, the screen was repeated to ensure that the PCR did not fail. If the result was still negative, then additional genomic DNA was extracted from the promising cell lines, and subjected to PCR using both the primer pairs. Initially, 100 pools of $G418^r$ + $GANC^r$ colonies were screened. From this, 10 of the pools looked promising because they gave negative results for the primer pair that was detecting the 380 bp region (Figures 35 and 36). However, upon re-isolation of genomic DNA and PCR with both pairs of primers, a number of samples that initially looked promising were now giving positive signals for the 380 bp region. In Figure 37, samples 4, 17, 54 and 57 now appear to be positive when they were initially negative. One explanation for this could be the notion that these are pools of cells. Initially, a pool may have contained a vast majority of the desired cell line, with only a very small percentage of parental 293 cells. Thus, when genomic DNA was first isolated and screened, it would appear to be negative for the presence of the 380 bp region. It could be the case, however, that the desired cell line with the 380 bp deletion has a reduced growth rate as compared to the parental 293 cells, and thus when genomic DNA was again isolated from the cells at a later date, the pool may have become overgrown by the parental 293 cell line. In this case, when the PCR was again performed, the pool would test positive for the presence of the 380 bps. There have been examples in literature where recovery of clonal

cell lines from complex pools is extremely difficult due to clonal variablility in growth rates (Shichiri *et al.*, 1993; Hanson and Sedivy, 1995).

A number of different explanations exist for why the desired cell line may have a reduced growth rate as compared to the parental 293 cells. The decision to remove the enhancer region of the E1 promoter may play a role in this idea of clonal variability in growth rates. As previously stated, the enhancer and packaging sequences were removed from the targeting construct, while only the core elements of the promoter remained. Essentially, by deleting the first 380 bps from the Ad genome, only the TATA box element of the promoter remained. This should be sufficient to drive expression of the E1 region; however, the level of expression will be greatly reduced. In deletion studies of the E1 region, it was found that deleting the enhancer region from bp 194 to bp 358 resulted in mRNA levels that were 0.07 of the wild-type level (reviewed in Berk, 1986). Thus, the level of E1A expression will be greatly reduced, and this may lead to altered morphology and growth characteristics. When E1 expression was completely eliminated in 293 cells through the use of antisense E1A, the cells were unable to survive and underwent senescence (Quinlan, 1993). The expression of the E1A region in 293 cells is clearly required for immortilization, and lowering the level of E1A expression may affect the growth characteristics of the cells. Therefore, the desired cell line which contained a deletion of the enhancer region may have exhibited reduced growth when compared to the parental 293 cell line.

Based on this idea of mixed populations of cells and variable growth rates, the 10 pools that initially tested negative for the 380 bps were deemed to be promising pools. In order to isolate out the cell line of interest, the pools were diluted to single cells and plated in a 150 mm plate at a density of 50 cells/plate. After two weeks, ten homogeneous foci from each plate were picked using cloning rings and expanded. Genomic DNA was isolated from the cells and again screened using the same procedure. From this round of screening, eight of the cell lines looked

promising due to the lack of a product when amplifying the 380 bp region (Figure 38). Additional genomic DNA was isolated from these pools, and screened using both sets of primers. As can be seen in Figure 39, all the samples which initially appeared promising were now giving rise to positive signals for the 380 bp region.

A number of explanations exist for why the pools initially appeared promising but then gave rise to positive signals. First, although the cells were treated with saline citrate to render them as single cells and thus homogeneous foci, many of the foci may have arose from more than one cell. It is difficult to separate single cells, and if the cells are under-treated by the chemical they may produce clumps, while if they are over-treated the viability of the cells will be reduced (Freshney, 2000). Therefore, although many of the cells appeared as single cells under the microscope, two or three cells may have been clumped together in some cases. This could then result in the same phenomena that was previously discussed, in which the desired cell line has reduced growth rates and the pool becomes overgrown by the parental 293 cells. Second, it is possible that either the cell lines became contaminated with wild-type 293 cells, or the PCR became contaminated with 293 cell genomic DNA. When performing the PCR, genomic 293 DNA was used as the positive control, which constitutes a risk for contamination. Kim *et al.* (1991) ran into this problem of contamination in their laboratory despite the use of a separate room, together with dedicated micro-pipettes and solutions, laminar flow hoods, gloves, caps and gowns for the setting up of PCR assays.

Due to the initial promising screening results, we feel confident that the cell line with the desired deletion is present in the pools of cells. Additional cloning and purification of the cell lines must be performed, with the utmost care taken to ensure that contamination does not occur. Once the cell line is isolated, it must then be characterized in terms of physiological characteristics, transformation efficiency, viral growth and stability. Once isolated and characterized, this cell line will prove invaluable for the growth and packaging of E1-deleted

vectors. Due to the lack of homology on the left-hand side of the transgene in most common E1 vectors, the cell line will be unable to undergo a double-crossing over event with the vector. Therefore, RCA will be prevented from occurring and the cell line can be used confidently and safely for the production of recombinant adenoviruses to be used in human gene therapy.

5. SUMMARY

In summary, based on the results obtained in this study, the following conclusions can be stated:

1) The left cellular-viral junction from 293 cells was cloned into the plasmid p293LJ, and the junction was sequenced.
2) The 293 cell line used in this study appears to contain a 3928 bp deletion in the PSG4 gene located at the cellular-viral junction.
3) The targeting vector PAM2 was constructed in order to modify the 293 cell line, and contained the left cellular-viral junction, the *neo* gene, the Ad5 genome from bp 381 to bp 3834, and the HSV-*tk* gene.
4) Preliminary results are promising that the desired cell line with the 380 bp deletion is present within the isolated cell lines, and further cloning and purification of the cell lnes must be performed in order to isolate the homogeneous cell line.

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